ABSTRACT

Methods, compositions and uses are provided for bispecific antibodies comprising one or more unnatural amino acids. The bispecific antibodies may bind to two or more different receptors, co-receptors, antigens, or cell markers on one or more cells. The bispecific antibodies may be used to treat a disease or condition (e.g., cancer, autoimmune disease, pathogenic infection, inflammatory disease). The bispecific antibodies may be used to modulate (e.g., stimulate or suppress) an immune response.
FIG. 1

X = pAcF
FIG. 8

Growth of MDA-MB-435/Her2+ cells and PBMC (n = 3)

FIG. 9

MDA-MB-435/Her2+ Tumor
FIG. 13

Ramos cytotoxicity

- αCD20/αCD3
- unconjugated

RFU vs. pM
FIG. 14

A549 cytotoxicity

- αEGFR/αCD3
- unconjugated

RFU vs. Concentration (pM)
FIG. 15

HT29 cytotoxicity

- αEGFR/αCD3
- unconjugated

RFU

Concentration (pM)
FIG. 20A

αCLL-1/αCD3 BiFab

Control
αCLL-1 Fab
αCLL-1/αCD3 BiFab

HL-60
CLL1+

Jurkat
CD3+

Fluorescence intensity

Count

FIG. 20B

αCD33/αCD3 BiFab

Control
αCD3 Fab
αCD33 Fab
αCD33/αCD3 BiFab

HL-60
CD33+

Jurkat
CD3+

Fluorescence intensity

Count
**FIG. 21A**

[Graph showing the relative viability of HL-60 cells with different concentrations of CL11+CD3 MIX and CL11-CD3 BiFab.]

**FIG. 21B**

[Graph showing the relative viability of HL-60 cells with different concentrations of CD33+CD3 MIX and CD33-CD3 BiFab.]
FIG. 23

- DTT

+ DTT
FIG. 25

A431 cells

EC₅₀ = 54.5 pM

- Hu808-UCHT1 Bifab
- Unconjugated Hu808 and UCHT1

% Cytotoxicity

nM
BISPECIFIC ANTIBODIES AND USES THEREOF

CROSS-REFERENCE


STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with the support of the United States government under Contract number GM062159 by National Institutes of Health (NIH). The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] Described herein are immunoglobulin constructs comprising one or more unnatural amino acids, methods of making such constructs, pharmaceutical compositions and medicaments comprising such constructs, and methods of using such constructs and compositions to prevent, inhibit, and/or treat a disease or condition in a subject.

BACKGROUND OF THE INVENTION

[0004] Antibodies are natural proteins that the vertebrate immune system forms in response to foreign substances (antigens), primarily for defense against infection. For over a century, antibodies have been induced in animals under artificial conditions and harvested for use in therapy or diagnosis of disease conditions, or for biological research. Each individual antibody producing cell produces a single type of antibody with a chemically defined composition, however, antibodies obtained directly from animal serum in response to antigen inoculation actually comprise an ensemble of non-identical molecules (e.g., polyclonal antibodies) made from an ensemble of individual antibody producing cells.

[0005] Some immunoglobulin constructs, such as bispecific antibodies, may bind to two or more different antigens. A number of recombinant strategies have been developed to synthesize bispecific antibodies, which include single chain variable fragment (scFv)-derived formats such as diabodies, tandem diabodies, BiIes (bispecific T-cell engager), and DART’s (Dual Affinity Re-Targeting), as well as immunoglobulin G (IgG)-based formats such as Triomab, DVI-Ig (Dual Variable Domain antibodies), and two-in-one antibodies. In addition, a number of chemical approaches have been developed which largely exploit the reactivity of lysine or cysteine residues within the antibody. Another chemical strategy has been reported in which heterodimeric peptides with a branched azidothione linker were fused to the antibody in a site-specific manner. These current approaches may result in heterogeneous products and/or may require the development of antigen-specific ligands, rather than utilizing the diverse pool of existing selective, high affinity monoclonal antibodies.

[0006] Disclosed herein are immunoglobulin constructs comprising one or more unnatural amino acids, methods of producing such constructs, and uses thereof.

SUMMARY OF THE INVENTION

[0007] Disclosed herein are bispecific antibodies comprising: an anti-CD3 antibody or anti-CD3 antibody fragment, a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically connected to the second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may be site-specifically connected to the second antibody or antibody fragment by one or more linkers. The second antibody or antibody fragment may comprise an anti-CD8 or FvIII antibody. The second antibody or antibody fragment may comprise an anti-Her2 antibody. The second antibody or antibody fragment may comprise an anti-CS1 antibody. The second antibody or antibody fragment may comprise an anti-CLL-I antibody. The second antibody or antibody fragment may comprise an anti-CD20 antibody. The second antibody or antibody fragment may comprise an anti-CDR1 antibody. The second antibody or antibody fragment may comprise an anti-CD44v6 antibody. The secondary antibody or antibody fragment may comprise an anti-FvIII antibody. The secondary antibody or antibody fragment may comprise an anti-CD8 or FvIII antibody. The secondary antibody or antibody fragment may comprise an anti-CDR1 antibody. The secondary antibody or antibody fragment may comprise an anti-CDR1 antibody.

[0008] The bispecific antibody may be of a Formula I: X-L1‘-Y or a Formula IIA: Y-L1‘-X, wherein: X comprises the anti-CD3 antibody or anti-CD3 antibody fragment; L1‘ comprises the one or more linkers; and Y comprises the second antibody or antibody fragment. X may comprise a human antibody or human antibody fragment. X may comprise a humanized antibody or humanized antibody fragment. X may comprise a human antibody or human antibody fragment. X may comprise a fully human antibody or fully human antibody fragment. X may comprise a chimeric antibody or portion thereof. X may comprise a cross-species reactive antibody or a portion thereof. The anti-CD3 antibody may be cross-species reactive with human and cynomolgus monkey. X and/or Y may comprise one or more Fv, X and/or Y may comprise one or more Fv, X and/or Y may comprise one or more Fv, X and/or Y may comprise one or more Fv.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Figure 1 is a schematic showing the structure of an exemplary form of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0010] Disclosed herein are bispecific antibodies comprising: an anti-CD3 antibody or anti-CD3 antibody fragment, a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically connected to the second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may be site-specifically connected to the second antibody or antibody fragment by one or more linkers. The second antibody or antibody fragment may comprise an anti-CD8 or FvIII antibody. The second antibody or antibody fragment may comprise an anti-Her2 antibody. The second antibody or antibody fragment may comprise an anti-CS1 antibody. The second antibody or antibody fragment may comprise an anti-CLL-I antibody. The second antibody or antibody fragment may comprise an anti-CD20 antibody. The second antibody or antibody fragment may comprise an anti-CDR1 antibody. The second antibody or antibody fragment may comprise an anti-CD44v6 antibody. The secondary antibody or antibody fragment may comprise an anti-FvIII antibody. The secondary antibody or antibody fragment may comprise an anti-CD8 or FvIII antibody. The secondary antibody or antibody fragment may comprise an anti-CDR1 antibody. The secondary antibody or antibody fragment may comprise an anti-CDR1 antibody.
bodies, CDR1, CDR2, CDR3, combinations of CDR’s, variable regions, framework regions, constant regions, heavy chains, light chains, and variable regions, alternative scaffold non-antibody molecules. X and/or Y may comprise a Fab fragment. The anti-CD3 antibody fragment may be UCHT1. Y may comprise at least a portion of a Fab fragment. Y may comprise at least a portion of an antibody or antibody fragment that binds to an antigen on a myeloid cell. Y may comprise at least a portion of an antibody or antibody fragment that binds to an antigen on a lymphocyte. Y may comprise at least a portion of an antibody or antibody fragment that binds to an antigen on a B-cell or D-cell progenitor. Y may comprise at least a portion of an antibody or antibody fragment that binds to an antigen on a cancerous cell. Y may comprise at least a portion of an antibody selected from the group comprising an anti-CD20 antibody, an anti-EGFRvIII antibody, an anti-CS1 antibody, an anti-CD19 antibody, and an anti-CD33 antibody. Y may comprise at least a portion of a Fab fragment of an antibody selected from the group comprising an anti-CD20 antibody, an anti-EGFRvIII antibody, an anti-CS1 antibody, an anti-CD19 antibody, and an anti-CD33 antibody. Y may comprise at least a portion of a Fab fragment of an antibody selected from the group comprising an anti-ROR1 antibody, an anti-CD44v6 antibody, an anti-IVL antibody, an anti-II.13Rabbit antibody and an anti-bseWtue1 antibody. Y may comprise at least a portion of a Fab fragment of an antibody selected from the group comprising an anti-ROR1 antibody, an anti-CD44v6 antibody, an anti-IVL antibody, an anti-II.13Rabbit antibody and an anti-bseWtue1 antibody. Y may comprise at least a portion of an anti-Her2 antibody. Y may comprise at least a portion of a Fab fragment of an anti-Her2 antibody. Y may be selected form SEQ ID NOS: 3-16.

[0009] The bispecific antibody may further comprise a second linker. The bispecific antibody may be of Formula II: X-L1-L2-Y or Formula III: Y-L2-L1-X, wherein: A L1 is coupled to X to produce a first intermediate of Formula III: X-L1 or Formula IIIA: L1-X, wherein X comprises an anti-CD3 antibody or an anti-CD3 fragment antibody; and L1 comprises a first linker before being coupled to X; L2 is coupled to Y to produce a second intermediate of Formula IV: Y-L2 or Formula IV A: L2-Y, wherein Y comprises at least a portion of a second antibody or antibody fragment and L2 comprises a second linker before being coupled to Y; and the first intermediate is coupled to the second intermediate. L1 may comprise one or more chemical groups selected from an alkyoxoamine, hydrazine, aryl azide, alkyll azide, alkyne, alkene, tetrazine, dichlorotriaze, tetrylate, succinimidyl carbonate, benzos triazole carbonate, nitrophenyl carbonate, trichlorotriazine, carbonylation, succinimidyl carbonate, maleimide, vinyl sulfone, haloacetamide, cyclooctyne, trans-cyclooctane, cyclopropane, norborene, and disulfide. L2 may comprise one or more chemical groups selected from an alkyoxoamine, hydrazine, aryl azide, alkyll azide, alkyne, alkene, tetrazine, dichlorotriaze, tetrylate, succinimidyl carbonate, benzos triazole carbonate, nitrophenyl carb onate, trichlorotriazine, carbonylation, succinimidyl carbonate, maleimide, vinyl sulfone, haloacetamide, cyclooctyne, trans-cyclooctene, cyclopropane, norborene, and disulfide. One terminus of L1 may comprise an alkyoxoamine. One terminus of L2 may comprise an alkyoxoamine. One terminus of L1 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise an azide group. One terminus of L2 may comprise a cyclooctyne group. One terminus of L2 may comprise an azide group.
derivatives, glycine derivatives, ring-substituted phenylalanine and tyrosine derivatives, linear core amino acids, diamino acids, D-amino acids, N-methyl amino acids, or a combination thereof. The site-specific connection may occur via the one or more unnatural amino acids of X. The site-specific connection may occur via the one or more unnatural amino acids of Y.

[0011] The purity of the bispecific antibody may be equal to or greater than 50%. The purity of the bispecific antibody may be equal to or greater than 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97% or 98%. The homogeneity of the bispecific antibody may be equal to or greater than 50%. The homogeneity of the bispecific antibody may be equal to or greater than 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97% or 98%.

[0012] Further disclosed herein are methods of producing bispecific antibodies, the method comprising connecting a plurality of anti-CD3 antibodies or anti-CD3 antibody fragments to a plurality of second antibodies or antibody fragments to produce a plurality of anti-CD3 bispecific antibodies, wherein at least 60% of the anti-CD3 bispecific antibodies are identical.

[0013] Disclosed herein are methods of producing a bispecific antibodies of Formula II: X-L1"-L2"-Y or Formula IIa: Y-L2"-L1"-X, comprising: coupling L1" to X to produce a first intermediate of Formula III: X-L1' or Formula IIIa: L1'-X, wherein X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; and L1" comprises a first linker before being coupled to X, coupling L2" to Y to produce a second intermediate of Formula IV: Y-L2'-Y, or Formula IVa: L2'-Y, wherein Y comprises a portion of a second antibody or antibody fragment and L2" comprises a second linker between being coupled to Y; and linking the first intermediate to the second intermediate, thereby producing the antibody of Formula II or HA. Coupling L1" to X may comprise site-specific coupling of L1" to X. Coupling L2" to Y may comprise site-specific coupling of L2" to Y. The method may further comprise incorporating one or more unnatural amino acids into X. The method may further comprise incorporating one or more unnatural amino acids into Y. Incorporating one or more unnatural amino acids into X and/or Y may comprise modifying one or more amino acid residues in X and/or Y to produce one or more amber codons in X and/or Y. The one or more unnatural amino acids may be incorporated into X and/or Y in response to an amber codon. The one or more unnatural amino acids may be site-specifically incorporated into X and/or Y. Incorporating one or more unnatural amino acids may comprise the use of one or more tRNA synthetases. The tRNA synthetase may be an aminocetyl tRNA synthetase. The tRNA synthetase may be a mutant tRNA synthesis incorporating one or more unnatural amino acids may comprise a tRNA/anti-tRNA synthetase pair. The tRNA/tRNA synthetase pair may comprise a tRNA/aminoacyl-tRNA synthetase pair. The tRNA/tRNA synthetase pair may comprise a tRNA/aminoacyl-tRNA synthetase pair.

[0014] Coupling L1" to X may occur at the one or more unnatural amino acids in X. Coupling L2" to Y may occur at the one or more unnatural amino acids in Y. Coupling L1" to X and coupling L2" to Y may occur sequentially. Coupling L1" to X and coupling L2" to Y may occur simultaneously. Coupling L1" to X may occur prior to coupling L2" to Y. Coupling L2" to Y may occur prior to coupling L1" to X. Coupling L1" to X and/or coupling L2" to Y may comprise forming one or more oxime bonds between L1" and X and/or between L2" and Y. Coupling L1" to X and/or coupling L2" to Y may comprise forming one or more covalent bonds between L1" and X and/or between L2" and Y. Coupling L1" to X and/or coupling L2" to Y may comprise forming one or more non-covalent bonds between L1" and X and/or between L2" and Y. Coupling L1" to X and/or coupling L2" to Y may comprise forming one or more ionic bonds between L1" and X and/or between L2" and Y.

[0015] Linking the first intermediate to the second intermediate may comprise a Huisgen-cycloaddition. Linking the first intermediate to the second intermediate may comprise a Diels-Halder reaction. Linking the first intermediate to the second intermediate may comprise a hetero Diels-Alder reaction. Linking the first intermediate to the second intermediate may comprise an enzyme-mediated reaction. Linking the first intermediate to the second intermediate may comprise a copper-free [3+2] Huisgen-cycloaddition reaction. Linking the first intermediate to the second intermediate may produce an oxime. Linking the first intermediate to the second intermediate may produce a hetero Diels-Alder adduct. Linking the first intermediate to the second intermediate may produce a nucleophilic substitution reaction product. Linking the first intermediate to the second intermediate may produce an amide. Linking the first intermediate to the second intermediate may produce a carbamate. Linking the first intermediate to the second intermediate may produce a thioether. Linking the first intermediate to the second intermediate may produce a Michael reaction product. Linking the first intermediate to the second intermediate may produce a cross-linking reaction product. Linking the first intermediate to the second intermediate may produce a metathesis reaction product. Linking the first intermediate to the second intermediate may produce a disulfide bridge. Linking the first intermediate to the second intermediate may produce an oxidative coupling product. Linking the first intermediate to the second intermediate may produce an acyl-transfer reaction product. Linking the first intermediate to the second intermediate may produce a photo click reaction product. Linking the first intermediate to the second intermediate may produce a maleimide bridge. The distance between X and Y may be less than or equal to 50, 45, 40, 35, 30, 25, or 20 angstroms (Å). The distance between X and Y may be greater than or equal to 5 angstroms (Å).

[0016] Further disclosed herein are pharmaceutical compositions comprising a bispecific antibody, wherein the bispecific antibody comprises an anti-CD3 antibody or anti-CD3 antibody fragment; a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically connected to the second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may be site-specifically connected to the second antibody or antibody fragment by one or more linkers. The anti-CD3 antibody or anti-CD3 antibody fragment may
comprise one or more unnatural amino acids. The second antibody or antibody fragment comprises one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The pharmaceutical composition may further comprise a pharmaceutically acceptable diluent, a pharmaceutically acceptable excipient or a pharmaceutically acceptable carrier.

[0017] Disclosed herein are methods for treating a disease or condition in a subject in need thereof, comprising administering a bispecific antibody, wherein the bispecific antibody comprises an anti-CD3 antibody or anti-CD3 antibody fragment; a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is specifically connected to the second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may be site-specifically connected to the second antibody or antibody fragment by one or more linkers. The antibody or antibody fragment may comprise one or more unnatural amino acids. The second antibody or antibody fragment may comprise one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The disease or condition may be a cancer. The cancer may be a breast cancer. The cancer may be a glioma or glioblastoma. The cancer may be a multiple myeloma. The cancer may be an acute myeloblastic leukemia (AML). The cancer may be selected from an acute lymphoblastic leukemia (ALL), a B-cell chronic lymphocytic leukemia (B-CLL) and a mantle cell lymphoma (MCL). The disease or condition may be a pathogenic infection. The disease or condition may be an inflammatory disease, an autoimmune disease or a metabolic disease. The bispecific antibody or pharmaceutical composition may be administered by parenteral administration. The parenteral administration may comprise intravenous administration, subcutaneous administration, intraperitoneal administration, intramuscular administration, intravenous administration, intrathecal administration, intravertebral administration, or infusion. The bispecific antibody or pharmaceutical composition may be administered by a microneedle device. The bispecific antibody or pharmaceutical composition may be administered by topical, oral, or nasal administration.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The foregoing summary, as well as the following detailed description of the disclosure, may be better understood when read in conjunction with the appended figures. For the purpose of illustrating the disclosure, shown in the figures are embodiments which are presently preferred. It should be understood, however, that the disclosure is not limited to the precise arrangements, examples and instrumentalities shown.

[0019] The invention may be best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to the drawings, the various features of the drawings are not to scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0020] FIG. 1 depicts a ribbon diagram of a UCHT1-Fab fragment.

[0021] FIG. 2A depicts the structure of p-acetylphenylalanine.

[0022] FIG. 2B depicts the structure of bifunctional ethylene glycol linkers.

[0023] FIG. 2C depicts a general scheme for the generation of bispecific antibodies.

[0024] FIGS. 3A-E depict ESI-MS analysis of Fab fragments before linker and after linker conjugation. FIG. 3A depicts Herceptin Fab mutant (LS02X, X=pAcF); FIG. 3B depicts Herceptin Fab-(PEG)4-Az; FIG. 3C depicts Herceptin Fab-(PEG)4-Oct; FIG. 3D depicts UCHT1 Fab mutant (HK138X, X=pAcF); and FIG. 3E depicts UCHT1 Fab-(PEG)4-Oct.

[0025] FIG. 3F-J depicts deconvoluted mass spectrum of Fab fragments before and after linker conjugation. FIG. 3F depicts Herceptin Fab mutant (LS02X, X=pAcF); FIG. 3G depicts Herceptin Fab-(PEG)4-Az; FIG. 3H depicts Herceptin Fab-(PEG)4-Oct; FIG. 3I depicts UCHT1 Fab mutant (HK138X, X=pAcF); and FIG. 3J depicts UCHT1 Fab-(PEG)4-Oct.

[0026] FIGS. 4A-B depict an SDS-PAGE gel (FIG. 4A) and size exclusion chromatography FPLC trace (FIG. 4B) of the anti-HER2/anti-CD3 heterodimer.

[0027] FIG. 5A-D depict bispecific binding of anti-HER2/anti-CD3 Fab heterodimer. FACS-based binding assay with Jurkat (CD3+) cells (FIG. 5A) and SK-BR-3 (HER2+) cells (FIG. 5B). FIG. 5C-D depicts fluorescence microscopic images of the interaction between SK-BR3 cells (larger, dark grey circles, highlighted by the vertical arrows in FIG. 5C) and Jurkat cells (smaller, light grey circles, highlighted by the horizontal arrows in FIG. 5C-D) in the presence of the conjugated anti-HER2/anti-CD3 heterodimer (FIG. 5C) or in the presence of a mixture of unconjugated anti-HER2 Fab and anti-CD3 Fab at a 1:1 ratio (FIG. 5D).

[0028] FIG. 6 depicts a graph of dose-dependent cytotoxicity with MDA-MB-435/HER2+ cells in the presence of human PBMCs and antibody heterodimer.

[0029] FIG. 7A-D depict microscopic images from a cytotoxicity assay. FIG. 7A depicts microscopic images from a cytotoxicity assay with HER2+ cells treated with a Fab mixture; FIG. 7B depicts microscopic images from a cytotoxicity assay with HER2+ cells treated with a bispecific antibody heterodimer; FIG. 7C depicts microscopic images from a cytotoxicity assay with HER2+ cells treated with a Fab mixture; FIG. 7D depicts microscopic images from a cytotoxicity assay with HER2+ cells treated with a bispecific antibody heterodimer.

[0030] FIG. 8 depicts growth curves of MDA-MB-435/HER2+ cells premixed with hPBMC and Matrigel. The ratios of the cancer cells to hPBMCs were 1:1 (square), 1:2 (triangle) and 1:5 (x).

[0031] FIG. 9 depicts a graph of the tumor volume of a MDA-MB-435/HER2+ tumor treated with a bispecific antibody (circle), Fab mixture (square) or PBS (triangle).

[0032] FIG. 10 depicts IVIS imaging of two heterodimer treated mice (left two) and two unconjugated Fab treated mice (right two).

[0033] FIG. 11 depicts excised tumors or MatriGel from a preventative anti-HER2/anti-CD3 Fab heterodimer study. The top row is the heterodimer treated group (n=7). The middle row is the unconjugated Fabs treated group (n=7). The bottom row is the PBS-treated group (n=6).

[0034] FIG. 12 shows a general scheme for synthesizing bifunctional linkers.

[0035] FIG. 13 depicts a graph of the cytotoxicity of Ramos cells treated with an anti-CD20/anti-CD3 bispecific antibody or treated with unconjugated Fab fragments.
[0036] FIG. 14 depicts a graph of the cytotoxicity of A549 cells treated with an anti-EGFR/anti-CD3 bispecific antibody or treated with unconjugated Fab fragments.

[0037] FIG. 15 depicts a graph of the cytotoxicity of HT29 cells treated with an anti-EGFR/anti-CD3 bispecific antibody or treated with unconjugated Fab fragments.

[0038] FIG. 16 depicts a graph of the cytotoxicity of AGS cells treated with an anti-EGFR/anti-CD3 bispecific antibody or treated with unconjugated Fab fragments.

[0039] FIG. 17A depicts the antigen binding fragments (Fab's) of anti-CS1 (hntLu3) and anti-CD3 (UCHT1) antibodies that were individually expressed in E. coli with pAcF suppressing a TAG mutation (at Serine 202) purified and conjugated using the optimized heterobifunctional linkers in FIG. 17B.

[0040] FIG. 17B depicts heterobifunctional linkers. The Tet-TEG-ONH₂ linker was conjugated to CS1 using the aminoxy functionality of the linker and the ketone of the pAcF incorporated at Serine 202 in an oxime ligation. The TCO-TEG-ONH₂ was conjugated to was conjugated to UCHT1 using the amine functionality of the linker and the ketone of pAcF incorporated at Serine 202 in an oxime ligation. The two proteins were subsequently “clicked” together to create the BiFab shown in FIG. 17A.

[0041] FIG. 17C shows the cytotoxicity of the BiFab, unconjugated monomers, and unconjugated monomers mixed was assayed using freshly purified, crude, human peripheral blood monocytes (PBMCs) against MM.1S cells in an overnight assay. Cytotoxicity results were readout by an LDH assay following standard protocols.

[0042] FIG. 18 depicts exemplary linkers.

[0043] FIG. 19A shows masses of CS1 Fab S202pAcF and UCHT1 Fab S202pAcF prior to conjugation to their respective linkers.

[0044] FIG. 19B shows masses of CS1 Fab S202pAcF and UCHT1 Fab S202pAcF conjugated to the Tet-TEG-ONH₂ (tet) and TCO-Tet-ONH₂ (TCO) linkers respectively.

[0045] FIG. 19C shows the mass of the CS1×UCHT1 biFab.

[0046] FIG. 20A shows selective binding of anti CLL-1/anti-CD3 BiFab towards CLL-1+HL-60 cells.

[0047] FIG. 20B shows selective binding of anti-CD3/anti-CD3 BiFab towards CD3+HL-60 cells.

[0048] FIG. 21A shows cytotoxicity of anti-CLL-1/anti-CD3 biFab towards CLL-1+HL-60 cells.

[0049] FIG. 21B shows cytotoxicity of anti-CD3/anti-CD3 biFab towards CD3+HL-60 cells.

[0050] FIG. 22A shows HL-60 IFN gamma release due to anti-CD3/anti-CD3 biFab or anti-CLL-1/anti-CD3 biFab.

[0051] FIG. 22B shows HL-60 IL-2 release due to anti-CD3/anti-CD3 biFab or anti-CLL-1/anti-CD3 biFab.

[0052] FIG. 23 shows expression and purification of UCHT1/anti-EGFR/III biFab. Antigen binding fragments (Fab's) of anti-EGFR/EGFR/III (hu806) and anti-CD3 (UCHT1) antibodies were individually expressed in E. coli with pAcF suppressing a TAG mutation (at Serine 202 LC, histidine for mouse HC, UCHT1) purified and conjugated using optimized heterobifunctional linkers.

[0053] FIG. 24A shows masses of Hu806 Fab S202pAcF and UCHT1 Fab S202pAcF prior to conjugation to their respective linkers.

[0054] FIG. 24B shows masses of Hu806 Fab S202pAcF and UCHT1 Fab S202pAcF conjugated to the Tet-TEG-ONH₂ and TCO-Tet-ONH₂ linkers respectively.

[0055] FIG. 24C shows the mass of the Hu806-UCHT1 BiFab.

[0056] FIG. 25 shows cytotoxicity of the Hu806-UCHT1 BiFab, unconjugated monomers, and unconjugated monomers mixed was assayed using freshly purified activated human peripheral blood monocytes (PBMCs) against A431 cells in an overnight assay. Cytotoxicity results were readout by an LDH assay following standard protocols.

DETAILED DESCRIPTION OF THE INVENTION

[0057] Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular methods or compositions. Each such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0058] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0059] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein may be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0060] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present.
invention. Any recited method may be carried out in the order of events recited or in any other order which is logically possible.

[0061] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the peptide" includes reference to one or more peptides and equivalents thereof, e.g., polypeptides, known to those skilled in the art, and so forth.

[0062] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0063] Disclosed herein are immunoglobulin constructs or bispecific antibodies. As used herein, the terms "immunoglobulin," "immunoglobulin construct" and "bispecific antibody" are used interchangeably. Generally, the bispecific antibody comprises one or more unnatural amino acids. The bispecific antibody may comprise an anti-CD3 antibody or anti-CD3 antibody fragment and a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment are site-specifically connected.

[0064] The bispecific antibody may comprise an anti-CD3 antibody or anti-CD3 antibody fragment and an anti-EGFRVIII antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the anti-EGFRVIII antibody or antibody fragment are site-specifically connected. The anti-CD3 antibody or anti-CD3 antibody fragment and/or the anti-EGFRVIII antibody or antibody fragment may comprise one or more unnatural amino acids.

[0065] The bispecific antibody may comprise an anti-CD3 antibody or anti-CD3 antibody fragment and an anti-Her2 antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the anti-Her2 antibody or antibody fragment are site-specifically connected. The anti-CD3 antibody or anti-CD3 antibody fragment and/or the anti-Her2 antibody or antibody fragment may comprise one or more unnatural amino acids.

[0066] The bispecific antibody may comprise an anti-CD3 antibody or anti-CD3 antibody fragment and an anti-CS-1 antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the anti-CS-1 antibody or antibody fragment are site-specifically connected. The anti-CD3 antibody or anti-CD3 antibody fragment and/or the anti-CS-1 antibody or antibody fragment may comprise one or more unnatural amino acids.

[0067] The bispecific antibody may comprise an anti-CD3 antibody or anti-CD3 antibody fragment and an anti-CLL-1 antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the anti-CLL-1 antibody or antibody fragment are site-specifically connected. The anti-CD3 antibody or anti-CD3 antibody fragment and/or the anti-CLL-1 antibody or antibody fragment may comprise one or more unnatural amino acids.

[0068] The bispecific antibody may comprise an anti-CD3 antibody or anti-CD3 antibody fragment and an anti-CD3 antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the anti-CD3 antibody or antibody fragment are site-specifically connected. The anti-CD3 antibody or anti-CD3 antibody fragment and/or the anti-CD3 antibody or antibody fragment may comprise one or more unnatural amino acids.

[0069] Further disclosed herein is a bispecific antibody of Formula I: X-L1-Y or Formula IIA: Y-L1-X, wherein (a) X comprises at least a portion of an anti-CD3 antibody or anti-CD3 antibody fragment; (b) L1 is a linker; and (c) Y comprises at least a portion of a second antibody or antibody fragment, wherein X is site-specifically linked to Y by the linker. X may comprise one or more unnatural amino acids. Y may comprise one or more unnatural amino acids. X and Y may comprise one or more unnatural amino acids.

[0070] Further disclosed herein is a bispecific antibody of Formula II: X-L1-L2-Y or an antibody of Formula IIA: Y-L2-L1-X, wherein (a) X comprises at least a portion of an anti-CD3 antibody or anti-CD3 antibody fragment; (b) L1 and L2 are linkers; and (c) Y comprises at least a portion of a second antibody or antibody fragment, wherein X is site-specifically linked to Y by the linkers. X may comprise one or more unnatural amino acids. Y may comprise one or more unnatural amino acids. X and Y may comprise one or more unnatural amino acids.

I. Antibodies and Antibody Fragments

[0071] The bispecific antibodies disclosed herein (e.g., an antibody of Formula I, Formula IIA, Formula II, Formula IIA) may bind to two or more different antigens. The bispecific antibody may comprise an antibody, antibody fragment, or combination thereof. The bispecific antibody may comprise an immunoglobulin-immunoglobulin (Ig-Ig) construct, wherein X comprises an immunoglobulin and Y comprises an immunoglobulin. The bispecific antibody may comprise an immunoglobulin-Fab (Ig-Fab) construct, wherein X comprises an immunoglobulin and Y comprises a Fab fragment. The bispecific antibody may comprise a Fab-Fab construct, wherein X comprises a Fab fragment and Y comprises a Fab fragment. As depicted by Formula IIA, L1, and HA, X and Y may be linked by one or more linkers (e.g., L1, L2).

[0072] As used herein, the term "antibody fragment" may refer to any form of an antibody other than the full-length form. Antibody fragments herein include antibodies that are smaller components that exist within full-length antibodies, and antibodies that have been engineered. Antibody fragments include, but are not limited to, Fv, Fab, Fab and Fab’2, single chain Fv (scFv), diabodies, triabodies, tetrabodies, bifunctional hybrid antibodies, CDR1, CDR2, CDR3, combinations of CDR’s, variable regions, framework regions, constant regions, heavy chains, light chains, alternative scaffold non-antibody molecules, and bispecific antibodies. Unless specifically noted otherwise, statements and claims that use the term "antibody" or "antibodies" may generally include "antibody fragment" and "antibody fragments."

[0073] The antibodies disclosed herein may be human, fully human, humanized, human engineered, non-human, and/or chimeric. For example, the antibody of Formula I may be a humanized antibody. In another example, the antibody of Formula II is a chimeric antibody. The antibodies disclosed herein may be based on or derived from human, fully human, humanized, human engineered, non-human and/or chimeric antibodies. For example, X and/or Y of Formula IIA may be based on or derived from a human engineered antibody. Alternatively, X and/or Y of Formula IIA may be based on or derived from a non-human antibody. Non-human antibody may be humanized to reduce immunogenicity to humans,
while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which CDRs (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally also comprises at least a portion of a human constant region. In some embodiments, some portions of a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the CDR residues are derived), e.g., to restore or improve antibody specificity or affinity.


[0075] Chimeric antibodies may refer to antibodies created through the joining of two or more antibody genes which originally encoded for separate antibodies. A chimeric antibody may comprise at least one humanized antibody from a first antibody and at least one amino acid from a second antibody, wherein the first and second antibodies are different. The antibodies disclosed herein may comprise antibody sequences from two or more different antibodies. For example, X of the antibodies disclosed herein (e.g., antibody of Formula I, IA, II or HA) may be from a first antibody and Y of the antibodies disclosed herein (e.g., antibody of Formula I, IA, II or IA) may be from a second antibody, wherein the first and second antibodies are different. In another example, X of the antibodies disclosed herein (e.g., antibody of Formula I, IA, II or HA) may be a chimeric antibody. Alternatively, or additionally, Y of the antibodies disclosed herein may be a chimeric antibody. The two or more different antibodies may be from the same species. For example, the species may be a bovine species, human species, or murine species. The two or more different antibodies may be from the same type of animal. For example the two or more different antibodies may be from a cow. The two or more different antibodies may be from a human. Alternatively, the two or more different antibodies may be from different species. For example, the two or more different antibodies are from a human species and a bovine species. In another example, the two or more different antibodies are from a bovine species and a non-bovine species. In another example, the two or more different antibodies are from a human species and a non-human species.

[0076] The anti-CD3 antibody or anti-CD3 antibody fragment may comprise at least a portion of a sequence selected from SEQ ID NOs: 1-2. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise a sequence that is at least 50% identical to a sequence selected from SEQ ID NOs: 1-2. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise a sequence that is at least 70% identical to a sequence selected from SEQ ID NOs: 1-2. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise a sequence that is at least 80% identical to a sequence selected from SEQ ID NOs: 1-2. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise a sequence that is at least 90% identical to a sequence selected from SEQ ID NOs: 1-2. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise a sequence that is at least 95% identical to a sequence selected from SEQ ID NOs: 1-2. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise a sequence that is at least 95% identical to a sequence selected from SEQ ID NOs: 1-2.
The second antibody or antigen fragment may comprise a sequence comprising five or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. The second antibody or antigen fragment may comprise a sequence comprising 6, 7, 8, 9, 10 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. The second antibody or antigen fragment may comprise a sequence comprising 15, 16, 17, 18, 19, 20 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. The second antibody or antigen fragment may comprise a sequence comprising 25, 30, 35, 40, 45, 50 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. The second antibody or antigen fragment may comprise a sequence comprising 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. The amino acids may be non-consecutive.

The antibodies disclosed herein may be based on or derived from an antibody or antigen fragment from a mammal, bird, fish, amphibian or reptile. Mammals include, but are not limited to, carnivores, rodents, elephants, marsupials, rabbits, bats, primates, seals, anteaters, cetaceans, odd-toed ungulates and even-toed ungulates. The mammal may be a human, non-human primate, mouse, sheep, cat, dog, cow, horse, goat, or pig. The mammal may be a human. The mammal may be a cynomolgus monkey.

Birds include, but are not limited to, albatrosses, hummingbirds, eagles, ostriches, cardinals, kiwis, and penguins. Fish may be cartilaginous fishes, ray-finned fishes, or lobe-finned fishes. Amphibians may include, but are not limited to, newts, salamanders, frogs and toads. Examples of reptiles include, but are not limited to, turtles, squamates, crocodiles and tuatars. Squamates may include amphisbaenians, lizards and snakes.

IA. Antibody or Antibody Fragments of X

The bispecific antibodies of Formulas I, IA, II, and/ or IIa disclosed herein may comprise X, wherein X comprises at least a portion of an anti-CD3 antibody or anti-CD3 antibody fragment comprising one or more unnatural amino acids. X may comprise an entire anti-CD3 antibody. The anti-CD3 antibody may be UC1T1. X may comprise at least a portion of an anti-CD3 antibody. X may comprise at least a portion of a monoclonal anti-CD3 antibody. X may comprise at least a portion of a CD3 polyclonal antibody. X may comprise at least a portion of a multivalent anti-CD3 antibody.

X may comprise an anti-CD3 antibody. X may comprise at least a portion of a Fab fragment of an anti-CD3 antibody. X may comprise an antibody fragment of an anti-CD3 antibody. The portion of the antibody may comprise an antibody fragment. Antibody fragments include, but are not limited to, Fv, Fab, F(ab)2, single chain Fv (scFv), diabodies, triabodies, tetrabodies, bifunctional hybrid antibodies, CDR1, CDR2, CDR3, combinations of CDR's, variable regions, framework regions, constant regions, heavy chains, light chains, alternative scaffold non-antibody molecules, and bispecific antibodies. X may comprise at least a portion of a heavy chain (HC) of an anti-CD3 antibody. X may comprise at least a portion of a light chain (LC) of a anti-CD3 antibody. X may comprise at least a portion of a variable region of an anti-CD3 antibody. X may comprise at least a portion of a constant region of anti-CD3 antibody. X may comprise an antibody or antibody fragment that binds to a portion of CD3. The portion of CD3 may comprise a CD3 gamma chain or a portion thereof. The portion of CD3 may comprise a CD3 delta chain or a portion thereof. The portion of CD3 may comprise a CD3 epsilon chain or a portion thereof. The portion of CD3 may comprise a combination of a CD3 gamma chain, a CD3 delta chain and a CD3 epsilon chain.
sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments. X may comprise a sequence that is at least about 95% homologous to a sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments. The sequence may be a peptide sequence. Alternatively, the sequence is a nucleotide sequence.

[00088] X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 20, 17, 15, 12, 10, 8, 6, 5, 4 or fewer amino acids. X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 4 or fewer amino acids. X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 3 or fewer amino acids. X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 2 or fewer amino acids. X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 1 or fewer amino acids. The amino acids may be consecutive, nonconsecutive, or a combination thereof. For example, X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 3 consecutive amino acids. Alternatively, or additionally, X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 2 non-consecutive amino acids. In another example, X may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 5 amino acids, wherein 2 of the amino acids are consecutive and 2 of the amino acids are non-consecutive.

[00089] X may be encoded by a nucleotide sequence that differs from a nucleotide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4 or fewer nucleotides or base pairs. X may be encoded by a nucleotide sequence that differs from a nucleotide sequence based on or derived from one or more anti-CD3 antibodies and/or anti-CD3 antibody fragments by less than or equal to about 5 amino acids, wherein 2 of the amino acids are consecutive and 2 of the amino acids are non-consecutive.

[00090] The peptide sequence of X may differ from the peptide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by one or more amino acid substitutions. The peptide sequence of X may differ from the peptide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by two or more amino acid substitutions. The peptide sequence of X may differ from the peptide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by three or more amino acid substitutions. The peptide sequence of X may differ from the peptide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by four or more amino acid substitutions. The peptide sequence of X may differ from the peptide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by five or more amino acid substitutions.
more nucleotide and/or base pair substitutions. The nucleotide sequence of X may differ from the nucleotide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by three or more nucleotide and/or base pair substitutions. The nucleotide sequence of X may differ from the nucleotide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by five or more nucleotide and/or base pair substitutions. The nucleotide sequence of X may differ from the nucleotide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by six or more nucleotide and/or base pair substitutions. The nucleotide sequence of X may differ from the nucleotide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by nine or more nucleotide and/or base pair substitutions. The nucleotide sequence of X may differ from the nucleotide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by twelve or more nucleotide and/or base pair substitutions. The nucleotide sequence of X may differ from the nucleotide sequence of the anti-CD3 antibody or anti-CD3 antibody fragment that it is based on and/or derived from by twenty or more nucleotide and/or base pair substitutions.

[0096] Y may comprise at least a portion of a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 50% identical to a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 60% identical to a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 70% identical to a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 80% identical to a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 90% identical to a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 95% identical to a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence that is at least 97% identical to a sequence selected from SEQ ID NOs: 3-16.

[0097] Y may comprise a sequence comprising five or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence comprising 6, 7, 8, 9, 10 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence comprising 15, 16, 17, 18, 19, 20 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence comprising 25, 30, 35, 40, 45, 50 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. Y may comprise a sequence comprising 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids based on or derived from a sequence selected from SEQ ID NOs: 3-16. The amino acids may be non-consecutive.

[0098] Y may comprise at least a portion of an antibody. The antibody may be an anti-CD20 antibody. The antibody may be an anti-EGFR antibody. The anti-EGFR antibody may be an anti-Her1 antibody. The anti-EGFR antibody may be an anti-Her2 antibody. The anti-EGFR antibody may be an anti-Her3 antibody. The anti-EGFR antibody may be an anti-Her4 antibody. The antibody may be an anti-EGFR antibody wherein the anti-EGFR antibody specifically binds a mutated EGFR. The mutated EGFR may be EGFRVIII.

[0099] The antibody may bind a cell adhesion molecule. The antibody may be an anti-CS1 antibody. The anti-CS1 antibody may comprise cetuximab, also referred to as HuLuc63. The anti-CS1 antibody may be humanized. The antibody may bind a receptor. The antibody may be an anti-CLL-1 antibody. The antibody may be an anti-CD33 antibody. The antibody may be an anti-ROR antibody. The ROR antibody may be an anti-ROR1 antibody. The antibody may be an anti-CD44 antibody. The anti-CD44 antibody may be an anti-CD44v6 antibody. The antibody may be an anti-PVRL4 antibody. The antibody may be an anti-interleukin receptor (ILR) antibody. The interleukin receptor antibody may bind to an II.R selected from an II.1R, an II.2R, an II.3R, an II.4R, an II.5R, an II.6R, an II.7R, an II.8R, an II.9R, an II.10R, an II.11R, an II.12R, an II.13R, an II.14R, an II.15R
and an II.17. The interleukin receptor antibody may bind to II.13R02. The interleukin receptor antibody may be an anti-II.13R02 antibody.

- **[0100]** The antibody may be a bispecific single chain (bsc) antibody. The bsc antibody may be a bsuWae-1 antibody.

- **[0101]** Y may comprise a Fab fragment of an antibody. Y may comprise a Fab fragment of an anti-H receptor antibody. Y may comprise a Fab fragment of an anti-CD20 antibody. Y may comprise a Fab fragment of an anti-EGFR antibody. Y may comprise a Fab fragment of an anti-H receptor antibody. Y may comprise a Fab fragment of an anti-CD52 antibody. Y may comprise a Fab fragment of an anti-CD3 antibody. Y may comprise a Fab fragment of an anti-RO1 antibody. Y may comprise a Fab fragment of an anti-CD44v6 antibody. Y may comprise a Fab fragment of an anti-PVRL4 antibody. Y may comprise a Fab fragment of an anti-II.13R02 antibody.

- **[0102]** Y may comprise an antibody or antibody fragment that binds to at least a portion of a receptor on a cell. Y may comprise an antibody or antibody fragment that binds to at least a portion of a co-receptor on a cell. Y may comprise an antibody or antibody fragment that binds to at least a portion of a receptor on a B cell. Y may comprise an antibody or antibody fragment that binds to at least a portion of an antigen or cell surface marker on a cell. The cell may be a hematopoietic cell. The hematopoietic cell may be a myeloid cell. The myeloid cell may be an erythrocyte, thrombocyte, neutrophil, monocyte, macrophage, eosinophil, basophil, or mast cell. The cell may be a lymphoid cell. The hematopoietic cell may be a lymphoid cell. The lymphoid cell may be a B cell, T cell, or NK cell. The hematopoietic cell may be a leukemia cell. The lymphoid cell may be a lymphocyte. The hematopoietic cell may be a lymphocyte. The cell may be a cancerous cell. The cell may be a tumor cell. The cell may be a leukemic cell. The cell may be a non-tumor cell. The cell may be a prostate cell. The cell may be a breast cell. The cell may be a glial cell. The cell may be a liver cell, kidney cell, lung cell, cardiac cell, muscle cell, nerve cell, neuron, brain cell, epithelial cell, or osophageal cell.

- **[0103]** Y may comprise an antibody or antibody fragment that binds to a receptor on a cell. The receptor may be a growth factor receptor. The growth factor receptor may be an epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) or fibroblast growth factor receptor (FGFR). The EGFR may be EGFR1. The EGFR may be EGFR2. The EGFR may be EGFR3 or EGFR4. The EGFR may have a mutation. The EGFR may be EGFRvIII. The receptor may be an interleukin receptor (IL). The interleukin receptor may be selected from IL1, an IL2, an IL3, an IL4, an IL5, an IL6, an IL7, an IL8, an IL9, an IL10, an IL11, an IL12, an IL13, an IL14, an IL15 and an IL17. The interleukin receptor may be IL13R02.

- **[0104]** Y may comprise an antibody or antibody fragment that binds to an antigen or cell surface marker on a hematopoietic cell. Y may comprise an antibody or antibody fragment that binds to an antigen or cell surface marker on a B cell. The antigen or cell surface marker on the B cell may be a B-lymphocyte antigen CD20 (CD20).

- **[0105]** Y may comprise an antibody or antibody fragment that binds to differentiation antigen. The differentiation antigen may be a CD44. The CD44 may be CD44v6. The differentiation antigen may be CD33. CD33 may be highly expressed on cells of an acute myeloid leukemia. CD33 may be expressed on a cancer stem cell.

- **[0106]** Y may comprise an antibody or antibody fragment that binds to a cell surface protein. The cell surface protein may be a cell adhesion molecule. The cell adhesion molecule may be a cell surface glycoprotein. The cell surface glycoprotein may be CS1, also referred to as CD3 subset 1, CRACC, SLAMF7, CD3, and 19A24. CS1 may comprise a sequence with CD138. The cell adhesion molecule may be PVRL4, also referred to as Nectin 4.

- **[0107]** Y may comprise an antibody or antibody fragment that binds to C-type lectin-like molecule-1. Y may comprise an antibody or antibody fragment that binds a receptor tyrosine kinase-like orphan receptor (ROR). The ROR may be ROR1. Y may comprise a bispecific single chain monoclonal antibody. The bispecific single chain monoclonal antibody may be bsuWae-1.

- **[0108]** Y may comprise an antibody or at least a portion of an antibody that is a human, fully human, humanized, human engineered, non-human, or chimeric antibody. Y may comprise an antibody or at least a portion of an antibody that is a mammalian antibody. Y may comprise an antibody or at least a portion of an antibody that is a non-mammalian antibody.

- **[0109]** Y may comprise a sequence based on or derived from one or more antibodies and/or antibody fragments sequences. Y may comprise a sequence that is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, 99% or more homologous to a sequence based on or derived from one or more antibodies and/or antibody fragments. Y may comprise a sequence that is at least about 70% homologous to a sequence based on or derived from one or more antibodies and/or antibody fragments. Y may comprise a sequence that is at least about 80% homologous to a sequence based on or derived from one or more antibodies and/or antibody fragments. Y may comprise a sequence that is at least about 90% homologous to a sequence based on or derived from one or more antibodies and/or antibody fragments. Y may comprise a sequence that is at least about 95% homologous to a sequence based on or derived from one or more antibodies and/or antibody fragments. The sequence may be a peptide sequence. Alternatively, the sequence is a nucleotide sequence.

- **[0110]** Y may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more antibodies or antibody fragments by less than or equal to about 20, 17, 15, 12, 10, 8, 6, 5, 4 or fewer amino acids. Y may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more antibodies or antibody fragments by less than or equal to 4 or fewer amino acids. Y may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more antibodies or antibody fragments by less than or equal to about 2 or fewer amino acids. Y may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more antibodies or antibody fragments by less than or equal to about 1 or fewer amino acids. The amino acids may be consecutive, nonconsecutive, or a combination thereof. For example, Y may comprise a peptide sequence that differs from a peptide sequence based on or derived from one or more antibodies or antibody fragments by less than or equal to about 3 consecutive amino acids.
Alternatively, or additionally, Y may comprise a peptide sequence that differs from a polynucleotide sequence by less than about 2 non-consecutive nucleotides. In another example, Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than about 5 nucleotides or base pairs, wherein 2 of the nucleotides or base pairs are consecutive and 2 of the nucleotides are non-consecutive.

[0111] Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 15 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 12 or fewer nucleotides or base pairs.

Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 9 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 7 or fewer nucleotides or base pairs.

Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 6 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 4 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 3 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 2 or fewer nucleotides or base pairs.

Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 1 or fewer nucleotides or base pairs. Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 0 or fewer nucleotides or base pairs.

Y may comprise a polynucleotide sequence that differs from a polynucleotide sequence by less than or equal to about 5 or fewer nucleotides or base pairs, wherein 2 of the nucleotides or base pairs are consecutive and 2 of the nucleotides or base pairs are non-consecutive.

[0112] The peptide sequence of Y may differ from the polynucleotide sequence of the antibody or antigen fragment by less than or equal to about 5 nucleotides or base pairs. The peptide sequence of Y may differ from the polynucleotide sequence of the antibody or antigen fragment by less than or equal to about 10 nucleotides or base pairs.
[0116] The distance between X and Y may be between about 1 angstroms (Å) to about 120 angstroms (Å). The distance between X and Y may be between about 5 angstroms (Å) to about 105 angstroms (Å). The distance between X and Y may be between about 10 angstroms (Å) to about 90 angstroms (Å). The distance between X and Y may be between about 10 angstroms (Å) to about 80 angstroms (Å). The distance between X and Y may be between about 10 angstroms (Å) to about 50 angstroms (Å). The distance between X and Y may be between about 25 angstroms (Å) to about 30 angstroms (Å). The distance between X and Y may be between about 15 angstroms (Å) to about 45 angstroms (Å). The distance between X and Y may be equal to or greater than about 4.5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 27, 30 or more angstroms. Each of these distances between X and Y may be equal to or greater than about 10 angstroms. The distance between X and Y may be equal to or greater than about 20 angstroms. The distance between X and Y may be equal to or less than about 110, 100, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30 or fewer angstroms. The distance between X and Y may be equal to or less than about 100 angstroms. The distance between X and Y may be equal to or less than about 80 angstroms. The distance between X and Y may be equal to or less than about 60 angstroms. The distance between X and Y may be equal to or less than about 40 angstroms.

II. Linkers

[0117] The bispecific antibodies disclosed herein may comprise one or more linkers (e.g., L1, L2). Unless otherwise specified, a linker may refer to a linker before it is coupled, reacted or linked to an antibody, antibody fragment, or another linker (e.g., L1, L2, L3), or a linker that is coupled, reacted or linked to an antibody or antibody fragment to produce an intermediate (e.g., X-L1-L1′-Y-L1, L1-L1′-Y-L1, L1-L1′-Y-L1, Y-L1, L1′-Y-L1-L1). The bispecific antibodies disclosed herein may comprise one or more linkers. The antibodies disclosed herein may comprise three or more linkers. The antibodies disclosed herein may comprise 4, 5, 6, 7 or more linkers. The linker may comprise one or more linkers. The linker may comprise a single intermediate. The linker may comprise a bispecific linker. The linker may be a bifunctional linker. The linker may be bifunctional. The linker may be bifunctional. The linker may be bifunctional. The linker may be bifunctional.

[0118] One or more linkers may be formed by reaction of an amino acid on X with a linker already attached to X. One or more linkers may be formed by reaction of an amino acid on Y with a linker already attached to Y. One or more linkers may be formed by reaction of a linker already attached to X with another linker already attached to Y. In order to form a linker already attached to X or Y, a bifunctional linker, with two orthogonally reactive functional groups, may be coupled to X or Y, such that one remaining reactive functional group is available for subsequent coupling.

[0120] The linker may be the product of a bioorthogonal reaction. The linker may comprise an oxime, a tetrazole, a Diels-Alder adduct, a hetero Diels-Alder adduct, an aromatic substitution reaction product, a nucleophilic substitution reaction product, an ester, an amide, a carbamate, an ether, a thioether, or a Michael reaction product. The linker may be a cycloaddition product, a metathesis reaction product, a metal-mediated cross-coupling reaction product, a radical polymerization product, an oxidative coupling product, an acyl-labeling reaction product, or a click reaction product. The cycloaddition may be a Huisgen-cycloaddition. The cycloaddition may be a copper-free [3+2] Huisgen-cycloaddition. The cycloaddition may be a Diels-Alder reaction. The cycloaddition may be a hetero Diels-Alder reaction. The linker may be the product of an enzyme-mediated reaction. The linker may be the product of a transglutaminase-mediated reaction. The linker may comprise a disulfide bridge that connects two cysteine residues, such as ThioBridge™ technology by PolyTherics. The linker may comprise a maleimide bridge that connects two amino acid residues. The linker may comprise a maleimide bridge that connects two cysteine residues.

[0121] Each of the one or more linkers may comprise one or more ethylene glycols. Each of the one or more linkers may comprise at least one ethylene glycol, for example, hydrazine, arylylalkyl azide, alkylene, alkene, tetrazine, dichlorotriazine, treyslate, succinimidyl carbonate, benzotriazole carbonate, nitrophenyl carbonate, tricholorphenyl carbonate, carbonylimidazole, succinimidyl succinate, maleimide, vinylsilifone, lucoxanthamide and disulfide. The alkene may be selected from norbornene, trans-cyclooctene, and cyclopentene. Each of the one or more linkers may comprise at least one ethylene glycol. Each of the one or more linkers may comprise one or more norbornene, trans-cyclooctene, and cyclopentene. Each of the one or more linkers may comprise at least one tetrazine. Each of the one or more linkers may comprise at least one tetrazine.

[0122] The linker may couple with one or more natural amino acids on X or Y. The linker may couple with one or more unnatural amino acids on X or Y. The linker may couple with an amino acid which is the product of site-specific mutagenesis. The linker may couple with a cysteine which is the product of site-specific mutagenesis. The linker (e.g., substituted maleimide) may couple with a cysteine which has the product of site-specific mutagenesis, as well as a native cysteine residue. Two linkers, each with complementary reactive functional groups, may couple with one another.

[0123] The one or more linkers may comprise a cleavable linker. The one or more linkers may comprise a non-cleavable linker. The one or more linkers may comprise a flexible linker. The one or more linkers may comprise an inflexible linker.

[0124] The one or more linkers may comprise an alkoxo-amino, azide group and/or cyclooctyne group at one or more termini. The one or more linkers may comprise an alkoxo-amino group at one terminus and an azide group at the other terminus. The one or more linkers may comprise an alkoxo-amino at one terminus and a cyclooctyne group at the other terminus.

[0125] The one or more linkers may be coupled to X, Y, or a combination thereof. The one or more linkers may be coupled to X and/or Y to form one or more intermediates of the formula III: X-L1-Y, Formula IIIA: X-L1, Formula IV: X-L1′-Y, Formula V: X-L2-Y.
L2'Y or Formula IVA: Y-L2'. The one or more linkers may be coupled to X and/or Y to form X-L1'-Y-L2'. L1'-X or L2'-Y, X-L1' may be coupled to Y: X-L1' may be coupled to L2'-Y. X-L1' may be coupled to Y. X may be coupled to L2'-Y: Y-L2' may be coupled to L1'-X. Y-L2' may be coupled to X. Y may be coupled to L1'-X. The one or more linkers may be coupled to X and/or Y by oxime formation. The one or more linkers may be coupled to X and/or Y by a cyclooctyne, cyclopropane, aryl/alkyl azides, trans-cyclooctene, norbornene, tetrazine bond, or a combination thereof. The one or more linkers may be coupled to X and/or Y by a covalent bond, noncovalent bond, ionic bond, or a combination thereof.

0126 The two or more linkers may be linked. The two or more linkers may be linked through one or more copper-free reactions. The two or more linkers may be linked through one or more cyclodadditions. The two or more linkers may be linked through one or more copper-free [3+2] Huisgen-cyclodadditions. The two or more linkers may be linked through one or more copper-containing reactions. The two or more linkers may be linked through one or more Diels-Alder reactions. The two or more linkers may be linked through one or more hetero Diels-Alder reactions.

0127 The linker may possess a length that is sufficiently long to allow the anti-CD3 antibody or antibody fragment and the second antibody or antibody fragment to be linked without steric hindrance from one another and sufficiently short to retain the intended activity of the antibody. The intended activity of the antibody may be to bring an effector cell and a target cell within a proximity sufficient to allow the targeting cell to have an effect on the target cell. The one or more linkers may be sufficiently hydrophilic that it does not cause instability of the antibody. The one or more linkers may be sufficiently hydrophilic that it does not cause insolubility of the antibody. The one or more linkers may be sufficiently stable. The one or more linkers may be sufficiently stable in vivo (e.g., it is not cleaved by serum, enzymes, etc.). The one or more linkers may be between about 1 angstroms (Å) to about 120 angstroms (Å) in length. The one or more linkers may be between about 5 angstroms (Å) to about 105 angstroms (Å) in length. The one or more linkers may be between about 1 angstroms (Å) to about 100 angstroms (Å) in length. The one or more linkers may be between about 5 angstroms (Å) to about 90 angstroms (Å) in length. The one or more linkers may be between about 10 angstroms (Å) to about 80 angstroms (Å) in length. The one or more linkers may be equal to or greater than about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 27, 30 or more angstroms in length. The one or more linkers may be equal to or greater than about 20 angstroms in length. The one or more linkers may be equal to or greater than about 40 angstroms in length. The one or more linkers may be equal to or less than about 60 angstroms in length. The one or more linkers may be equal to or less than about 40 angstroms in length.

0128 The total length of the linkers may be between about 1 angstroms (Å) to about 120 angstroms (Å). The total length of the linkers may be between about 5 angstroms (Å) to about 105 angstroms (Å). The total length of the linkers may be between about 10 angstroms (Å) to about 90 angstroms (Å). The total length of the linkers may be between about 20 angstroms (Å) to about 80 angstroms (Å). The total length of the linkers may be between about 40 angstroms (Å) to about 70 angstroms (Å). The total length of the linkers may be between about 50 angstroms (Å) to about 60 angstroms (Å). The total length of the linkers may be between about 60 angstroms (Å) to about 70 angstroms (Å). The total length of the linkers may be between about 70 angstroms (Å) to about 80 angstroms (Å). The total length of the linkers may be between about 80 angstroms (Å) to about 90 angstroms (Å). The total length of the linkers may be between about 90 angstroms (Å) to about 100 angstroms (Å). The total length of the linkers may be equal to or greater than about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 27, 30 or more angstroms. The total length of the linkers may be equal to or greater than about 20 angstroms. The total length of the linkers may be equal to or less than about 110, 100, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30 or fewer angstroms. The total length of the linkers may be equal to or less than about 100 angstroms. The total length of the linkers may be equal to or less than about 80 angstroms. The total length of the linkers may be equal to or less than about 60 angstroms. The total length of the linkers may be equal to or less than about 40 angstroms.

III. Unnatural Amino Acids

0129 The bispecific antibodies disclosed herein may comprise one or more unnatural amino acids. As used herein, the terms “unnatural amino acid” and “non-natural amino acid” may be used interchangeably and may refer to non-proteinogenic amino acids that either occur naturally or are chemically synthesized. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise X, wherein X comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise X, wherein X does not comprise an unnatural amino acid. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise X, wherein Y comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y does not comprise an unnatural amino acid. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y does not comprise an unnatural amino acid. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y does not comprise an unnatural amino acid. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y does not comprise an unnatural amino acid. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y comprises one or more unnatural amino acids. The bispecific antibodies of Formulas I, IA, I, and/or IA disclosed herein may comprise Y, wherein Y does not comprise an unnatural amino acid.
body or antibody fragment; or a combination thereof. The one or more unnatural amino acids may be incorporated into an antibody fragment of an antibody (e.g., antibody of Formula I, Ia, II, and HA; intermediate (e.g., intermediate of Formula III, IIIa, IV, IVa); X antibody or antibody fragment; Y antibody or antibody fragment; or a combination thereof. The one or more unnatural amino acids may be site-specifically incorporated into an antibody fragment of an antibody (e.g., antibody of Formula I, Ia, II, and HA); intermediate (e.g., intermediate of Formula III, IIIa, IV, IVa); X antibody or antibody fragment; Y antibody or antibody fragment; or a combination thereof. [0131] The one or more unnatural amino acids may be incorporated into a heavy chain, light chain, variable region, or constant region fragment of an antibody (e.g., antibody of Formula I, Ia, II, and HA); intermediate (e.g., intermediate of Formula III, IIIa, IV, IVa); X antibody or antibody fragment; Y antibody or antibody fragment; or a combination thereof. [0132] The one or more unnatural amino acids may be encoded by a codon that does not code for one of the twenty natural amino acids. The one or more unnatural amino acids may be encoded by a nonsense codon (stop codon). The stop codon may be an amber codon. The amber codon may comprise a UAG sequence. The stop codon may be an ochre codon. The ochre codon may comprise a UAA sequence. The stop codon may be an opal codon. The opal or amber codon may comprise a UGA sequence. The one or more unnatural amino acids may be encoded by a four-base codon. [0133] The one or more unnatural amino acids may be p-acetylphenylalanine (pAcPhe) or pAcPh. The one or more unnatural amino acids may be encoded by the group comprising p-acetylphenylalanine (pAcPhe), p-benzylphenylalanine (pBpF), p-propargyloxyphenylalanine (pPrF), p-isopropylphenylalanine (pIF), p-cyanophenylalanine (pCNF), p-carboxyethylphenylalanine (pEcNF), 3-(2-naphthyl)alanine (Npa), p-boronophenylalanine (pBol), pyrrolidinoalanine (pOlf), (8-hydroxyquinolin-3-yl)alanine (HQA), selencysteine, and (2,2'-bipyridin-5-yl)alanine (Bip5A). [0134] The one or more unnatural amino acids may be β-amino acids (β3 and β2), homo-amino acids, proline and pyrroic acid derivatives, 3-substituted alaniine derivatives, glycine derivatives, ring-substituted phenylalanine and tyrosine derivatives, linear core amino acids, diaminoc acids, N-methyl amino acids, or a combination thereof. [0135] Additional examples of unnatural amino acids include, but are not limited to, 1) substituted tyrosines and phenylalanines. The analogues such as O-methyl-L-tyrosine, p-amino-L-phenylalanine, 3-nitro-L-tyrosine, p-nitro-L-phenylalanine, m-methoxy-L-phenylalanine and p-isopropyl-L-phenylalanine; 2) amino acids with aryl azide and benzophenone groups that may be photo-cross-linked; 3) amino acids that have unique chemical reactivity including acetyl-L-phenylalanine and m-acetyl-L-phenylalanine, O-allyl-L-tyrosine, O-(2-propynyl)-L-tyrosine, p-ethylcarboxy-L-phenylalanine and p-(3-oxobutanoyl)-L-phenylalanine; 4) heavy-atom-containing amino acids for phasing in X-ray crystallography including p-iodo and p-bromo-L-phenylalanine; 5) the redox-active amino acid dihydroxy-L-phenylalanine; 6) glycosylated amino acids including b-N-acetylglucosamine-O-serine and a-N-acetylglucosamine-O-serine; 7) fluorescent amino acids with naphthyl, dansyl, and 7-aminoacoumarin side chains; 8) photoconvertable and photoisomerizable amino acids with azobenzene and nitrobenzyl Cys, Ser, and Tyr side chains; 9) the phosphotyrosine mimetic p-carboxymethyl-L-phenylalanine; 10) the glutamine homologue homoglutamine; and 11) 2-aminoaceto- natoic acid. [0136] Additional unnatural amino acids are disclosed in Lin et al. (Ann Rev Biochem. 79:413-44, 2010), Wang et al. (Angew Chem Int Ed. 44:34-66, 2005) and PCT application numbers PCT/US2012/039472, PCT/US2012/039468, PCT/US2007/088009, PCT/US2009/058668, PCT/US2007/089142, PCT/US2007/088011, PCT/US2007/0001485, PCT/US2006/040937, PCT/US2006/047822 and PCT/US2006/044062, all of which are incorporated by reference in their entirety. [0137] The one or more unnatural amino acids may comprise at least one oxime, carbonyl, dicarboxyl, hydroxylamine group or a combination thereof. The one or more unnatural amino acids may comprise at least one carbonyl, dicarboxyl, alkoxy-amine, hydrazine, acyclic alkene, acyclic alkyne, cyclooctyne, aryalkyl azide, arborne, cyclopropene, trans-cyclooctene, or tetrazine functional group or a combination thereof. The one or more unnatural amino acids may be incorporated into X and/or Y by methods known in the art. Cell-based or cell-free systems may be used to alter the genetic sequence of X and/or Y, thereby producing X and/or Y with one or more unnatural amino acids. Auxotrophic strains may be used in place of engineered RNA and synthetase. The one or more unnatural amino acids may be produced through selective reaction of one or more natural amino acids. The selective reaction may be mediated by one or more enzymes. In one non-limiting example, the selective reaction of one or more cysteines with formylglycine generating enzyme (FGGE) may produce one or more formylglycines. [0138] The one or more unnatural amino acids may take part in a chemical reaction to form a linker. The chemical reaction to form the linker may be a bioorthogonal reaction. The chemical reaction to form the linker may be a click chemistry. IV. Antibody Compositions [0139] Disclosed herein are compositions comprising one or more bispecific antibodies disclosed herein. The compositions may comprise a bispecific antibody comprising an anti-CD3 antibody or anti-CD3 antibody fragment and a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically connected to the second antibody or antibody fragment. The compositions may comprise a bispecific antibody comprising (a) an anti-CD3 antibody or anti-CD3 antibody fragment; (b) a second antibody or antibody fragment; and (c) one or more linkers, wherein the one or more linkers links the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically linked to the second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise one or more unnatural amino acids. The second antibody or antibody fragment may comprise one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The composition may further
comprise one or more pharmaceutically acceptable excipients. The composition may further comprise one or more solvents or diluents.

[0140] The composition may comprise a bispecific antibody of Formula I: X-L1-Y, wherein (i) X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; (ii) L1 comprises a linker; and (iii) Y comprises a second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise one or more unnatural amino acids. The second antibody or antibody fragment may comprise one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The composition may further comprise one or more pharmaceutically acceptable excipients. The composition may further comprise one or more solvents or diluents.

[0141] The composition may comprise a bispecific antibody of Formula Ia: Y-L1-X, wherein (i) X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; (ii) L1 comprises a linker; and (iii) Y comprises a second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise one or more unnatural amino acids. The second antibody or antibody fragment may comprise one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The composition may further comprise one or more pharmaceutically acceptable excipients. The composition may further comprise one or more solvents or diluents.

[0142] The composition may comprise a bispecific antibody of Formula II: X-L1-L2-Y, wherein (i) X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; (ii) L1 and L2 comprise a linker; and (iii) Y comprises a second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise one or more unnatural amino acids. The second antibody or antibody fragment may comprise one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The composition may further comprise one or more pharmaceutically acceptable excipients. The composition may further comprise one or more solvents or diluents.

[0143] The composition may comprise a bispecific antibody of Formula Ia: Y-L1-L2-X, wherein (i) X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; (ii) L1 and L2 comprise a linker; and (iii) Y comprises a second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise one or more unnatural amino acids. The second antibody or antibody fragment may comprise one or more unnatural amino acids. The anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment may comprise one or more unnatural amino acids. The composition may further comprise one or more pharmaceutically acceptable excipients. The composition may further comprise one or more solvents or diluents.

[0144] The term “pharmaceutically acceptable” as used herein, refers to a material that does not abrogate the biological activity or properties of the agents described herein, and is relatively nontoxic (i.e., the toxicity of the material significantly outweighs the benefit of the material). In some instances, a pharmaceutically acceptable material may be administered to an individual without causing significant undesirable biological effects or significantly interfering in a deleterious manner with any of the components of the composition in which it is contained.

[0145] Pharmaceutical compositions herein may be formulated using one or more physiologically acceptable carriers including excipients and auxiliaries which facilitate processing of the active agents into preparations which are used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. A summary of pharmaceutical compositions is found, for example, in Remington: The Science and Practice of Pharmacy, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995); Hoover, John E., Remington’s Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. 1973; Liberman, H. A. and Lachman, L., Eds., Pharmaceutical Dosage Forms, Marcel Dekker, New York, N.Y., 1980; and Pharmaceutical Dosage Forms and Drug Delivery Systems, Seventh Ed. (Lippincott Williams & Wilkins, 1999).

[0146] A pharmaceutical composition disclosed herein may further comprise a pharmaceutically acceptable diluent (s), excipient(s), or carrier(s). The pharmaceutical compositions may include other medicinal or pharmaceutical agents, carriers, adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure, and/or buffers. In addition, the pharmaceutical compositions also contain other therapeutically valuable substances.

[0147] A pharmaceutical composition disclosed herein may be administered to a subject by any suitable administration route, including but not limited to, parenteral (intravenous, subcutaneous, intraperitoneal, intramuscular, intravascular, intrathecal, intravitreal, infusion, or local), topical, oral, or nasal administration. A pharmaceutical composition disclosed herein may be administered to a subject by an intranasal administration. A pharmaceutical composition disclosed herein may be administered to a subject by a microneedle device. The microneedle device may be used to deliver a low dosage of the pharmaceutical composition due to the relatively high potency or efficacy of the antibody. The microneedle device may be a microneedle device as described in U.S. Pat. No. 7,416,541, Peters et al., Pharm Res. 29:1618-26 (2012), and Daddona et al., Pharm Res. 28:159-65 (2011).

[0148] Formulations suitable for intramuscular, subcutaneous, peritumoral, or intravenous injection may include physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqeous and non-aqueous carriers, diluents, solvents, or vehicles including water, ethanol, polyols (propylene glycol, polyethylene glycol, glycerol, cremophor and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity is maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. Formulations suitable for subcutaneous injection also contain optional additives such as preserving, wetting, emulsifying, and dispensing agents.

[0149] For intravenous injections, an active agent may be optionally formulated in aqueous solutions, preferably in pharmaceutically compatible buffers such as Hank’s solution, Ringer’s solution, or physiological saline buffer.
[0150] Parenteral injections optionally involve bolus injection or continuous infusion. Formulations for injection are optionally presented in unit dosage form, e.g., in ampoules or in multi dose containers, with an added preservative. The pharmaceutical composition described herein may be in a form suitable for parenteral injection as a sterile suspensions, solutions or emulsions in oily or aqueous vehicles, and contain formulation agents such as suspending, stabilizing and/or dispersing agents. Pharmaceutical formulations for parenteral administration include aqueous solutions of an active agent in water soluble form. Additionally, suspensions are optionally prepared as appropriate oily injection suspensions.

[0151] The pharmaceutical composition described herein may be in unit dosage forms suitable for single administration of precise dosages. In unit dosage form, the formulation may divided into unit doses containing appropriate quantities of an active agent disclosed herein. The unit dosage may be in the form of a package containing discrete quantities of the formulation. Non-limiting examples are packaged tablets or capsules, and powders in vials or ampoules. Aqueous suspension compositions may be packaged in single-dose non-reclosable containers. Alternatively, multiple-dose reclosable containers are used, in which case it is typical to include a preservative in the composition. By way of example only, formulations for parenteral injection are presented in unit dosage form, which include, but are not limited to ampoules, or in multi dose containers, with an added preservative.

V. Antibody Production Methods

[0152] Disclosed herein are methods of producing bispecific antibodies comprising connecting an anti-CD3 antibody or anti-CD3 antibody fragment and a second antibody or antibody fragment site-specifically. Disclosed herein are methods of producing the antibody of Formula I: X-L1-Y or Formula IA: Y-L1-X, comprising coupling L1’ to X to produce an intermediate of Formula III: X-L1’ or Formula IIIA: L1’-X, wherein X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; and coupling the intermediate to Y; wherein Y comprises at least a portion of a second antibody or antibody fragment; thereby producing the antibody of Formula I or IA.

[0153] Disclosed herein are methods of producing bispecific antibodies of Formula I: X-L1-Y or Formula IA: Y-L1-X, comprising coupling L1’ to Y to produce an intermediate of Formula IV: Y-L1’ or Formula IVA: L1’-Y, wherein Y comprises at least a portion of a second antibody or antibody fragment; and coupling the intermediate to X, wherein X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; thereby producing the antibody of Formula I or IA. The method may further comprise incorporating one or more unnatural amino acids into X and/or Y. The method may further comprise incorporating one or more unnatural amino acids into X and/or Y site-specifically. Coupling L1’ to X may comprise coupling L1’ to X site-specifically. Coupling L2’ to Y may comprise coupling L2’ to Y site-specifically. Coupling L1’ to X and coupling L2’ to Y may comprise coupling L1’ to X site-specifically and coupling L2’ to Y site-specifically. The method may further comprise coupling L1’/L2’ to Y at the one or more unnatural amino acids of Y. The method may further comprise coupling L1’ to X at the one or more unnatural amino acids of Y.

[0155] Disclosed herein are methods of coupling one or more first linkers to an anti-CD3 antibody or anti-CD3 antibody fragment to produce a first intermediate; coupling one or more second linkers to a second antibody or antibody fragment; and coupling the first intermediate to the second intermediate via the linkers. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise an unnatural amino acid. The second antibody or antibody fragment may comprise an unnatural amino acid. Coupling one or more first linkers to the anti-CD3 antibody or anti-CD3 antibody fragment to produce the first intermediate may comprise forming a stable oxime between the anti-CD3 antibody or anti-CD3 antibody fragment and the linker. Coupling one or more second linkers to the second antibody or antibody fragment to produce the second intermediate may comprise forming a stable oxime between the second antibody or antibody fragment and the linker. The oxime may be formed between an alkoxy-amine group of the linker and the unnatural amino acid. The oxime may be formed between an alkoxy-amine group of the linker and a ketone of the unnatural amino acid. The oxime may be formed between an alkoxy-amine group of the linker and a ketone of the unnatural amino acid, wherein the unnatural amino acid is p-acetylphenylalanine (p-AcP). The oxime may be between an alkoxy-amine group of the first linker and the unnatural amino acid of the anti-CD3 antibody or anti-CD3 antibody fragment. The oxime may be formed between an alkoxy-amine group of the first linker and the unnatural amino acid of the anti-CD3 antibody or anti-CD3 antibody fragment. The oxime may be formed between an alkoxy-amine group of the second linker and the unnatural amino acid of the second antibody. The first/second linker may be a bifunctional linker. The bifunctional linker may comprise an azide group. The bifunctional linker may comprise a cyclooctyne group. The azide group may be linked to the cyclooctyne group through a chemical reaction. The chemical reaction may be a copper-free [3+2] Huisgen cycloaddition (“Click” reaction). The first/second linker may be a heterobifunctional linker. The heterobifunctional linker may comprise two reactive functional groups selected from alkoxy-amine, hydrazine, aroylalkyl azide, alkene, alkene, tetrazine, dichlorotriazine, tresylate, succinimidyl carbonate, benzotriazole carbonate, nitrophenyl carbonate, trichlorophenyl carbonate, carbonylimidazole, succinimidyl succinorate, maleimide, vinylsulfone, haloucatamine, and disulfide.

[0156] The anti-CD3 antibody may not comprise an unnatural amino acid while the second antibody or antibody fragment does comprise an unnatural amino acid. Coupling the one or more linkers to the anti-CD3 antibody or anti-CD3 antibody fragment may comprise forming a stable oxime between the anti-CD3 antibody or anti-CD3 antibody fragment and one or more of the first linkers to form the first intermediate. The oxime may be formed between an alkoxy-amine group of the first linker and the natural amino acid of the anti-CD3 antibody or anti-CD3 antibody fragment. Cou-
pling one or more second linkers to the second antibody or antibody fragment to produce the second intermediate may comprise forming a stable oxime between the second antibody or antibody fragment and the one or more second linkers. The oxime may be formed between an alkoxy-amine group of the second linker and the unnatural amino acid of the second antibody or antibody fragment. The first/second linker may be a bifunctional linker. The bifunctional linker may comprise an azide group. The bifunctional linker may comprise a cyclooctyne group. The azide group may be linked to the cyclooctyne group through a chemical reaction. The chemical reaction may be a copper-free [3+2] Huisgen-cycloaddition (“Click” reaction). The first/second linker may be a heterobifunctional linker. The heterobifunctional linker may comprise two reactive functional groups selected from alkoxy-amine, hydrazine, aryl/alkyl azide, alkyne, alkene, tetrazine, dichlororazirane, triazole, succinimidyl carbonate, benzotriazole carbonate, nitrophenyl carbonate, trichlorophenyl carbonate, carbonylimidazole, succinimidyl succinate, maleimide, vinylsulfone, haloacetamide, and disulfide.

[0157] The anti-CD3 antibody or anti-CD3 antibody fragment may comprise an unnatural amino acid, while the second antibody does not comprise an unnatural amino acid. Coupling the one or more linkers to second antibody or antibody fragment may comprise forming a stable oxime between the second antibody or antibody fragment and one or more of the second linkers to form the second intermediate. The oxime may be formed between an alkoxy-amine group of the second linker and the unnatural amino acid. Coupling one or more first linkers to the anti-CD3 antibody or anti-CD3 antibody fragment to produce the first intermediate may comprise forming a stable oxime between the anti-CD3 antibody or antibody fragment and the one or more first linkers. The oxime may be formed between an alkoxy-amine group of the first linker and the unnatural amino acid. The first/second linker may be a bifunctional linker. The bifunctional linker may comprise an azide group. The bifunctional linker may comprise a cyclooctyne group. The azide group may be linked to the cyclooctyne group through a chemical reaction. The chemical reaction may be a copper-free [3+2] Huisgen-cycloaddition (“Click” reaction). The first/second linker may be a heterobifunctional linker. The heterobifunctional linker may comprise two reactive functional groups selected from alkoxy-amine, hydrazine, aryl/alkyl azide, alkyne, alkene, tetrazine, dichlororazirane, triazole, succinimidyl carbonate, benzotriazole carbonate, nitrophenyl carbonate, trichlorophenyl carbonate, carbonylimidazole, succinimidyl succinate, maleimide, vinylsulfone, haloacetamide, and disulfide.

[0158] Conjugating the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise one or more Huisgen-cycloadditions. Conjugating the anti-CD3 antibody or antibody fragment to the second antibody or antibody fragment may comprise one or more copper-free [3+2] Huisgen-cycloadditions. Conjugating the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise one or more Diels-Alder reactions. Conjugating the anti-CD3 antibody or antibody fragment to the second antibody or antibody fragment may comprise one or more hetero Diels-Alder reactions.

[0159] Conjugating the first intermediate to the second intermediate may comprise one or more Huisgen-cycloadditions. Conjugating the first intermediate to the second intermediate may comprise one or more copper-free [3+2] Huisgen-cycloadditions. Conjugating the first intermediate to the second intermediate may comprise one or more copper-containing reactions. Conjugating the first intermediate to the second intermediate may comprise one or more Diels-Alder reactions. Conjugating the first intermediate to the second intermediate may comprise one or more hetero Diels-Alder reactions.

[0160] Conjugating the one or more of the first linkers to the one or more of the second linkers may comprise one or more Huisgen-cycloadditions. Conjugating the one or more of the first linkers to the one or more of the second linkers may comprise one or more copper-free [3+2] Huisgen-cycloadditions. Conjugating the one or more of the first linkers to the one or more of the second linkers may comprise one or more copper-containing reactions. Conjugating the one or more of the first linkers to the one or more of the second linkers may comprise one or more Diels-Alder reactions. Conjugating the one or more of the first linkers to the one or more of the second linkers may comprise one or more hetero Diels-Alder reactions.

[0161] Further disclosed herein is a method of producing the bispecific antibody of Formula II: X-L1-L2-Y, comprising (a) coupling L1" to X to produce a first intermediate of Formula IIIA: X-L1-L2-Y wherein X comprises at least a portion of an anti-CD3 antibody or anti-CD3 antibody fragment; (b) coupling L2" to Y to produce a second intermediate of Formula IV: L2-Y, wherein Y comprises at least a portion of an antibody or antibody fragment; and (c) linking the first intermediate of Formula III to the second intermediate of Formula IV, thereby producing the antibody of Formula II. The method may further comprise incorporating one or more unnatural amino acids into X and/or Y.

VA. Incorporation of Unnatural Amino Acids

[0162] Incorporating one or more unnatural amino acids into an antibody or antibody fragment may comprise modifying one or more amino acid residues in an antibody or antibody fragment. Modifying the one or more amino acid residues in an antibody or antibody fragment may comprise mutating one or more nucleotides in the nucleotide sequence encoding the antibody or antibody fragment. Mutating the one or more nucleotides in the nucleotide sequence encoding the antibody or antibody fragment may comprise altering a codon encoding an amino acid to a nonsense codon.

[0163] Incorporating one or more unnatural amino acids into an antibody or antibody fragment may comprise modifying one or more amino acid residues in an antibody or antibody fragment to produce one or more amber codons in an antibody or antibody fragment.

[0164] The one or more unnatural amino acids may incorporated into an antibody or antibody fragment in response to an amber codon. The one or more unnatural amino acids may be site-specifically incorporated into an antibody or antibody fragment.

[0165] Incorporating one or more unnatural amino acids into an antibody or antibody fragment may comprise use of one or more genetically encoded unnatural amino acids with orthogonal chemical reactivity relative to the canonical twenty amino acids to site-specifically modify antibody fragments. Incorporating the one or more unnatural amino acids
may comprise use of an evolved tRNA/aminocycl-tRNA synthetase pair to site-specifically incorporate one or more unnatural amino acids at defined sites in the antibody or antibody fragment in response to one or more amber nonsense codon.


VB. Coupling of Linkers

[0167] The methods disclosed herein may comprise coupling one or more linkers to one or more antibodies, antibody fragment(s), or combinations thereof to produce one or more antibody-linker and/or antibody fragment-linker molecules. The methods may comprise coupling a first linker to a first antibody or antibody fragment to produce an antibody-linker molecule or antibody fragment-linker molecule. The methods may comprise coupling a second linker to a second antibody or antibody fragment to produce an antibody-linker molecule or antibody fragment-linker molecule.

[0168] Coupling of the one or more linkers to the antibody or antibody fragment may occur sequentially. Coupling of the one or more linkers to the antibody or antibody fragment may occur sequentially. Coupling of the one or more linkers to the antibody or antibody fragment may occur in a single reaction volume. Coupling of the one or more linkers to the antibody or antibody fragment may occur in two or more reaction volumes.

[0169] Coupling one or more linkers to an antibody or antibody fragment may comprise forming one or more oximes between the linker and the antibody or antibody fragment. Coupling one or more linkers to an antibody or antibody fragment may comprise forming one or more stable bonds between linker and the antibody or antibody fragment. Coupling one or more linkers to an antibody or antibody fragment may comprise forming one or more covalent bonds between linker and the antibody or antibody fragment. Coupling one or more linkers to an antibody or antibody fragment may comprise forming one or more non-covalent bonds between linker and the antibody or antibody fragment. Coupling one or more linkers to an antibody or antibody fragment may comprise forming one or more ionic bonds between linker and the antibody or antibody fragment.

VC. Linking Antibodies and Antibody Fragments

[0170] The methods may comprise linking the antibody, antibody fragment and/or intermediates thereof to produce a bispecific antibody comprising (a) an anti-CD3 antibody or anti-CD3 antibody fragment; (b) a second antibody or antibody fragment; and (c) one or more linkers, wherein the one or more linkers link the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment. The anti-CD3 antibody or anti-CD3 antibody fragment may comprise one or more unnatural amino acids. Linking the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise conducting one or more copper-free reactions. Linking the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise conducting one or more copper-containing reactions. Linking the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise one or more cycloadditions. Linking the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise one or more Huisgen-cycloadditions. Linking the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise one or more Diels-Alder reactions. Linking the anti-CD3 antibody or anti-CD3 antibody fragment to the second antibody or antibody fragment may comprise one or more Hetero Diels-Alder reaction.

VD. Purification of Antibodies

[0171] The methods may further comprise purifying the bispecific antibody comprising (a) an anti-CD3 antibody or antibody fragment comprising one or more unnatural amino acids; (b) a second antibody or antibody fragment; and (c) one or more linkers, wherein the one or more linkers link the anti-CD3 antibody or antibody fragment to the second antibody or antibody fragment. The methods may further comprise purifying one or more intermediates of the antibody or antibody fragment antibody or antibody fragment (e.g., antibody-linker or antibody fragment-linker). Purifying the antibody or intermediates may comprise removal of excess linkers, non-linked antibodies or non-linked antibody fragments. Purifying the antibody or intermediates may comprise use of one or more concentrator columns, electrophoresis, filtration, centrifugation, chromatography or a combination thereof. Chromatography may comprise size-exclusion chromatography. Additional chromatography methods include, but are not limited to, hydrophobic interaction chromatography, ion exchange chromatography, affinity chromatography, metal binding, immunoaffinity chromatography, and high performance liquid chromatography or high pressure liquid chromatography. Electrophoresis may comprise denaturing electrophoresis or non-denaturing electrophoresis.

[0172] Antibodies or intermediates may comprise one or more tags. The linkers may comprise one or more tags. The tags may be used to purify the antibodies or intermediates. The one or more tags may be cleaved by one or more proteases. Examples of tags include, but are not limited to, polyhistidine, FLAG, HA, c-myc, V5, chitin binding protein (CBP), maltose binding protein (MBP), and glutathione-S-transferase (GST).

[0173] The methods may further comprise lyophilization or ultracentrifugation of the antibodies or intermediates.

[0174] The purity of the bispecific antibody may be equal to or greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more. The purity of the antibody may be equal to or greater than 85%. The purity of the antibody may be equal to or greater than 90%. The purity of the antibody may be equal to or greater than 95%. The purity of the antibody may be equal to or greater than 97%.

[0175] The purity of the intermediate (e.g., antibody-linker, antibody fragment-linker,) may be equal to or greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more. The purity of the antibody may be equal to or greater than 85%. The purity of the antibody may be equal to or greater than
90%. The purity of the antibody may be equal to or greater than 95%. The purity of the antibody may be equal to or greater than 97%.

**[0176]** The homogeneity of the bispecific antibody may be equal to or greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more. The homogeneity of the antibody may be equal to or greater than 85%. The homogeneity of the antibody may be equal to or greater than 90%. The homogeneity of the antibody may be equal to or greater than 95%. The homogeneity of the antibody may be equal to or greater than 97%.

**[0177]** The homogeneity of the intermediate (e.g., antibody-linker, antibody fragment-linker,) may be equal to or greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more. The homogeneity of the antibody may be equal to or greater than 85%. The homogeneity of the antibody may be equal to or greater than 90%. The homogeneity of the antibody may be equal to or greater than 95%. The homogeneity of the antibody may be equal to or greater than 97%.

VI. Cells

**[0178]** The bispecific antibodies disclosed herein may bind to one or more receptors, co-receptors, antigens, or cell markers on one or more cells. The bispecific antibodies disclosed herein may comprise (a) an anti-CD3 antibody or antibody fragment and (b) a second antibody or antibody fragment, wherein (i) the anti-CD3 antibody or antibody fragment binds to or interacts with CD3 on a first cell; (ii) the second antibody or antibody fragment binds to or interacts with a receptor, co-receptor, antigen or cell marker on a second cell; or (iii) a combination of (i) and (ii), and wherein (iv) the anti-CD3 antibody or antibody fragment comprises one or more unnatural amino acids; (v) the second antibody or antibody fragment comprises one or more unnatural amino acids; or (vi) a combination of (iv) and (v). The first cell may be an effector cell. The effector cell may be a T cell. The second cell may be a target cell. The target cell may be a cancerous cell. The first cell and the second cell may be the same type of cell. The first cell and the second cell may be different cell types. Alternatively, the first cell and the second cell may be the same cell.

**[0179]** The one or more cells may be hematopoietic cells. Hematopoietic cells include, but are not limited to, basophilic myelocytes, basophils, B-cells, burst forming unit erythroid (BFU-E), burst forming unit megakaryocytes (BFU-Mk), colony forming unit basophils (CFU-Bas), colony forming unit erythroid (CFU-E), colony forming unit eosinophils (CFU-El), colony forming unit granulocytes (CFU-G), colony forming unit granulocyte-erythrocyte macrophage (CFU-GEM), colony forming unit granulocyte macrophage (CFU-GM), colony forming unit megakaryocyte (CFU-Mk), common.medianeric progenitor, common lymphoid progenitor cells, common myeloid progenitors, common myeloid/myeloid progenitors, double negative 1 (DN1) cells, DN2 cells, DN3 cells, DN4 cells, double-positive cells (DP cells), eosinophilic myelocytes, eosinophils, erythrocytes, lymphoid stem cells, lymphoid-related dendritic cells, macrophages, mast cells, megakaryocytes, memory B-cells, memory cells, memory T-cells, monoblasts, monocytes, myeloblasts, myeloid stem cells, myeloid-related dendritic cells, neutrophilic myelocytes, neutrophils, natural killer cells (NK-cells), natural killer T-cells (NKT-cells), platelets, plasma cells, plasma B cells, plasmacyctes, effector B cells, mature B cells, pro-B1-cells, pro-B2-cells, pro-B-cells, pro-erythroblasts, promonocytes, regulatory T-cells (Tregs), T-cells, T-helper (Th) cells, Th0 cells, Th1 cells, Th2 cells, Th3 cells, Th17 cells, BFU-E or CFU-E may refer to erythroid precursor cells that may differentiate into erythrocytes. CFU-E cells may be more developed than BFU-E cells. CFU-Eo may refer to developmental type of blood-forming cells that may develop into eosinophils. CFU-G may refer to a developmental type of blood-forming cells that may be a precursor of granulocytes. CFU-GEMM may refer to a pluripotent type of precursor cell in the lineage of blood-forming cells that may differentiate into granulocytes, erythrocytes, monocytes and/or macrophages. CFU-GM may refer to a pluripotent type of precursor cell in the lineage of blood-forming cells that may differentiate into granulocytes and/or macrophages. BFU-Mk, CFU-Mk or CFU-MEG may refer to precursor cells that may differentiate into megakaryocytes. CFU-Mk or CFU-MEG cells may be more developed than BFU-Mk cells.

**[0180]** The one or more cells may be from an organ or tissue. The organ may be a gland organ. The organ may be an organ of the digestive or endocrine system. The organ may be both an endocrine gland and a digestive organ. The organ may be derived from endoderm, ectoderm, primitive endoderm, or mesoderm. The organ may be an adrenal gland. In some cases, the adrenal gland comprises chromaffin cells or ganglion cells. Alternatively, the organ is an appendix, bladder, or brain. In some cases, the brain comprises neurons (e.g., nerve cells) or glial cells. Glial cells include, but are not limited to, astrocytes, oligodendrocytes, and ependymal cells. In some instances, the organ is an ear, esophagus, eye, or gallbladder. The gallbladder comprises choleystocytes. The organ may be a kidney. The kidney may comprise a kidney glomerulus parietal cell, kidney glomerulups podocyte, kidney proximal tubule brush border cell, Loop of Henle thin segment cell, thick ascending limb cell, kidney distal tubule cell, kidney collecting ductal cell, or interstitial kidney cell. In some instances, the organ is a large intestine and the large intestine may comprise enterocytes, goblet cells, cavedoated tuft cells, enterocodnereine cells, or ganglion neurons. The organ may be a liver. The liver may comprise parenchymal or non-parenchymal cells. Examples of parenchymal cells comprise hepatocytes. Non-parenchymal cells include, but are not limited to, sinusoidal endothelial cells, kupffer cells and hepatic stellate cells. In some instances, the organ is a lung, mouth, nose, parathyroid gland, pineal gland, pituitary gland, skin, small intestine, stomach, spleen, thymus, thyroid gland, trachea, uterus, or vermiform appendix. In some instances, the organ may be a heart. In some instances, the heart comprises cardiomocytes. In some instances, the organ is a muscle (e.g., heart muscle, skeletal muscle, smooth muscle, etc.). The muscle may comprise myocytes.

**[0181]** In some cases, the cells are from a tissue. The tissue may be a connective tissue, epithelial tissue, muscular tissue, or nervous tissue. Alternatively, the tissue is a bone, tendon (both referred to as musculoskeletal grafts), corneal, skin, heart valve, or vein.

**[0182]** Connective tissue may be a fibrous tissue and is often found throughout the body. Examples of connective tissues include, but are not limited to, connective tissue, fat tissue, dense fibrous tissue, cartilage, bone, blood, and lymph. Generally, connective tissue has three main components:
cells, fibers, and extracellular matrix, which may be embedded in the body fluids. Fibroblasts are often the cells responsible for the production of connective tissue. The interaction of the fibers, the extracellular matrix and the water, together, may form the pliable connective tissue as a whole. Connective tissue may make up a variety of physical structures including tendons and the connective framework of fibers in muscles, capsules and ligaments around joints, cartilage, bone, adipose tissue, blood and lymphatic tissue. Connective tissue (CT) may be classified into three subtypes: embryonic CT, proper CT, and special CT. The proper CT subtype may include dense regular CT, dense irregular CT, and loose CT. The special CT subtype may include cartilage, bone, adipose tissue, and connective tissues of the thymus. Connective tissue may be divided into three subtypes: embryonic CT, proper CT, and special CT. The proper CT subtype may include dense regular CT, dense irregular CT, and loose CT. The special CT subtype may be divided into three subtypes: embryonic CT, proper CT, and special CT. The proper CT subtype may include dense regular CT, dense irregular CT, and loose CT. The special CT subtype may include cartilage, bone, adipose tissue, and connective tissues of the thymus. Connective tissue may be divided into three subtypes: embryonic CT, proper CT, and special CT. The proper CT subtype may include dense regular CT, dense irregular CT, and loose CT. The special CT subtype may include cartilage, bone, adipose tissue, and connective tissues of the thymus.

[0183] Often connective tissues have distinct functions, characteristics, and compositions. The functions of connective tissues may include storage of energy, protection of organs, providing structural framework for the body, and connection of body tissues. The connective tissue may be characterized by cells that are spread through an extracellular fluid. In some instances, the connective tissue may comprise a ground substance, which is often clear, colorless, and viscous fluid containing glycosaminoglycans and proteoglycans. The ground substance may fix the bodywater and the collagen fibers in the intercellular spaces. Ground substance may also slow the spread of pathogens.

[0184] The connective tissue may be fibrous and the fibrous tissue may comprise distinct compositions and be localized to specific areas of the body. For example, collagenous fibers often contain alpha polypeptide chains and may be primarily localized to a tendon, ligament, skin, corneum, cartilage, bone, blood vessels, gut, and intervertebral disc. In another example, elastic fibers may comprise elastic microfibril and elastin and may be primarily localized to an extracellular matrix. Reticular fibers are another example of fibrous tissue and may be localized to the liver, bone marrow, or lymphatic organs.

[0185] However, not all types of connective tissues are fibrous. Examples of non-fibrous connective tissues are adipose tissue and blood. Adipose tissue may provide a "mechanical cushioning" to our body. Although there is often no dense collagen network in adipose tissue, groups of adipocytes are kept together by collagen sheets in order to keep fat tissue under compression in place (for example the sole of the foot).

[0186] Epithelia are tissues that may consist of closely apposed cells without intervening intercellular substances. Epithelia are often avascular, but epithelia may become "growth" on an underlying layer of vascular connective tissue. The connective tissues around the epithelium may be separated by a basement membrane. Epithelium may cover all free surfaces of the body. Epithelium may also line the large internal body cavities, where it is termed mesothelium. Furthermore, the internal surfaces of blood and lymph vessels may be lined by epithelium, here called endothelium. Epithelia are often classified on the basis of the number of cell layers and the shape of the cells in the surface layer. If there is only one layer of cells in the epithelium, it is designated simple. If there are two or more layers of cells, it is termed stratified. Cells in the surface layer may be described according to their height as squamous (scale- or plate-like), cuboidal or columnar.

[0187] Different types of epithelial tissues may have specialized functions and locations within the body. For example, pseudostatified columnar may function to remove dust and particles from airways and may have cilia. The pseudostratified columnar may line the respiratory passageways. The simple columnar may be involved in absorption and often line the uterus and most organs of the digestive tract. The simple cuboidal may be involved in secretion and absorption and may be localized to glands, kidney (tubules), and ovaries. The simple squamous may play a role in diffusion and filtration and may be localized to lungs, walls of capillaries and vessels. The stratified squamous may protect underlying cells and is often localized to the skin, throat, vagina, and mouth. The stratified cuboidal may be involved in protection and may line ducts of the mammary glands, sweat glands, and pancreas. The stratified columnar may be involved in protection and is often localized to the male urethra and vas deferens, and parts of the pharynx.

[0188] Muscular tissue is often a contractile tissue and may be derived from the mesodermal layer of embryonic germ cells. Muscle cells may contain contractile filaments that move past each other and change the size of the cell. They are classified as skeletal, cardiac, or smooth muscles. Skeletal muscle or "voluntary muscle" may be anchored by tendons (or by aperistaltic at a few places) to bone and may be used to effect skeletal movement such as locomotion and in maintaining posture. Smooth muscle or "involuntary muscle" is often found within the walls of organs and structures such as the esophagus, stomach, intestines, bronchi, uterus, urethra, bladder, blood vessels, and the artery pili in the skin (in which it controls creation of body hair). Cardiac muscle is also an "involuntary muscle" but may be more structurally similar to skeletal muscle, and is often found in the heart.

[0189] Cardiac and skeletal muscles are often "striated" in that they contain sarcomeres and are packed into highly regular arrangements of bundles. While skeletal muscles may be arranged in regular, parallel bundles, cardiac muscle often connects at branching, irregular angles (called intercalated discs). Striated muscle may contract and relax in short, intense bursts, whereas smooth muscle may sustain longer or even near-permanent contractions.

[0190] Skeletal muscle may be divided into several subtypes. Type I, slow oxidative, slow twitch, or "red" muscle is often dense with capillaries and may be rich in mitochondria and myoglobin, giving the muscle tissue its characteristic red color. It can carry more oxygen and sustain aerobic activity. Type II, fast twitch muscle, has three major kinds, Type IIa, Type IIx, and Type IIb. Type IIa is often aerobic and may be rich in mitochondria and capillaries and may appear red. Type IIx (also known as type IId), which is often less dense in mitochondria and myoglobin. Type IIb, which may be anaerobic, glycolytic, "white" muscle that is often even less dense in mitochondria and myoglobin.

[0191] Nervous tissue is one of four major classes of tissue. Nervous tissue is often the main component of the nervous system, the brain, spinal cord, and nerves, which may regulate and control body functions. Nervous tissue is often composed of neurons and the neuroglia cells. Neurons may transmit impulses. Neuronal cells may assist in propagation of the nerve impulse as well as provide nutrients to the neuron. Nervous tissue is often made of nerve cells that may come in many varieties, all of which may be distinctly characterized by the axon or long stem like part of the cell that sends action potential signals to the next cell.

[0192] Functions of the nervous system may include sensory input, integration, controls of muscles and glands, homeostasis, and mental activity. Nervous tissue may react to
stimuli and may conduct impulses to various organs in the body which often bring about a response to the stimulus. Nerve tissue (as in the brain, spinal cord and peripheral nerves that branch throughout the body) are often made up of specialized nerve cells called neurons. Neurons are easily stimulated and transmit impulses very rapidly. A nerve often comprises many nerve cell fibers (neurons) bound together by connective tissue. A sheath of dense connective tissue, the epineurium may surround the nerve. This sheath penetrates the nerve to form the perineurium which surrounds bundles of nerve fibers. Blood vessels of various sizes may be seen in the epineurium. The endoneurium, which consists of a thin layer of loose connective tissue, surrounds the individual nerve fibers.

The cell body may be enclosed by a cell (plasma) membrane and may have a central nucleus. Granules called Nissl bodies are often found in the cytoplasm of the cell body. Within the cell body, extremely fine neurofilaments may extend from the dendrites into the axon. The axon is often surrounded by the myelin sheath, which forms a whitish, non-cellular, fatty layer around the axon. Outside the myelin sheath may be a cellular layer called the neurilemma or sheath of Schwann cells. The myelin sheath together with the neurilemma is also known as the medullary sheath. This medullary sheath may be interrupted at intervals by the nodes of Ranvier.

Neurons may be classified both structurally and functionally. Structural classification may group neurons according to the number of processes extending from their cell body. Three major neuron groups often make up this classification: multipolar (polar—end, pole), bipolar and unipolar neurons. Multipolar neurons often have three or more processes. These are the most common neuron type in humans (more than 99% of neurons belong to this class) and the major neuron type in the CNS. Bipolar neurons are often spindle-shaped, with a dendrite at one end and an axon at the other. An example may be found in the light-sensitive retina of the eye. Unipolar neurons often comprise sensory neurons. Sensory neurons normally have only a single process or fibre which divides close to the cell body into two main branches (axon and dendrite).

The cells may also comprise hair follicles, hair cells, ear hair cells, hair stem cells, or cochlear cells. Hair cells are often the sensory receptors of both the auditory system and the vestibular system. The auditory hair cells may be located within the organ of Corti on a thin basilar membrane in the cochlea of the inner ear. Cochlear hair cells may come in two anatomically and functionally distinct types: the outer and inner hair cells.

The cells described herein may be diseased, infected, mutated or genetically modified.

The one or more cells may be a pathogenic cell. Pathogenic cells include, but are not limited to, bacteria, viruses, fungi, and protozoans. Examples of pathogens may include, but are not limited to, the bacteria, viruses, fungi, and protozoans disclosed herein.

VII. Receptors, Co-Receptors, Antigens and Cell Markers

The bispecific antibodies disclosed herein may bind to one or more receptors, co-receptors, antigens, or cell markers on one or more cells. The bispecific antibodies disclosed herein may comprise (a) an anti-CD3 antibody or antibody fragment and (b) a second antibody or antibody fragment, wherein (i) the anti-CD3 antibody or antibody fragment binds to or interacts with CD3 on a first cell; (ii) the second antibody or antibody fragment binds to or interacts with a receptor, co-receptor, antigen or cell marker on a second cell; or (iii) a combination of (i) and (ii), and wherein (iv) the anti-CD3 antibody or antibody fragment comprises one or more unnatural amino acids; (v) the second antibody or antibody fragment comprises one or more unnatural amino acids; or (vi) a combination of (iv) and (v). The first cell and the second cell may be the same type of cell. The first cell and the second cell may be different cell types. Alternatively, the first cell and the second cell may be the same cell.

For example, for a bispecific antibody of Formula 1, IA, II, and/or HA, X may comprise an antibody or antibody fragment that binds to CD3 or a portion thereof. Alternatively, or additionally, for an antibody of Formula 1, IA, II, and/or II.A, Y comprises an antibody or antibody fragment that may bind to a receptor, co-receptor, antigen, transmembrane protein or cell marker.

Examples of receptors, co-receptors, antigens or cell markers may include, but are not limited to, a receptor, co-receptor, antigen or cell marker on a hematopoietic cell, tissue cell, epithelial cell, mesothelial cell, dermal cell, endothelial cell, dendritic cell, vascular cell, stromal cell, neuron, cancer cell, bacteria, fungus, or virus. The receptors, co-receptors, antigens or cell markers may be selected from the group comprising CD19, CD20, CD22, CD25, CD30, CD40, CD56, CD64, CD70, CD74, CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNAV, PSLMA, Cripto, ED-8, TFEBF2, EphB2, EphA2, FAP, or integrin, mesothelin, EGFR, TAG-72, GD2, CAIX, and ST4.

An antigen may evoke the production of one or more antibodies. An antigen may refer to a molecule or molecular fragment that may be bound by a major histocompatibility complex (MHC) and presented to a T-cell receptor. The term “antigen” may also refer to an immunogen. An immunogen may provoke an adaptive immune response if injected on its own into a subject. An immunogen may induce an immune response by itself. An antigen may also refer to a hapten. A hapten may be a small molecule. Generally, a hapten may induce an immune response when attached to a larger carrier molecule, such as a protein. Antigens may be proteins or polysaccharides. Antigens may comprise parts (e.g., coats, capsules, cell walls, flagella, fibrin, and toxins) of bacteria, viruses, and other microorganisms. Lipids and nucleic acids may be antigenic when combined with proteins and polysaccharides. Antigens may include superantigens, T-dependent antigens and T-independent antigens.

Antigens may be exogenous antigens or endogenous antigens. Exogenous antigens are typically antigens that have entered the body from the outside, for example by inhalation, ingestion, or injection. Some antigens may start out as exogenous antigens, and later become endogenous (for example, intracellular viruses). Intracellular antigens may be released back into circulation upon the destruction of the infected cell, again. Endogenous antigens may be antigens that have been generated within previously-normal cells as a result of normal cell metabolism, or because of viral or intracellular bacterial infection.

Antigens may also include autoantigens. An autoantigen may be a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognized by the immune system of patients suffering from a specific autoimmune disease. These antigens should, under normal conditions, not be the target of the immune system, but, due to mainly genetic
and environmental factors, the normal immunological tolerance for such an antigen has been lost in these patients. Antigens may include tumor antigens. Tumor antigens or neoantigens may be antigens that are presented by MHC I or MHC II molecules on the surface of tumor cells. These antigens may sometimes be presented by tumor cells and never by the normal ones. In this case, they are called tumor-specific antigens (TSAs) and, in general, result from a tumor-specific mutation. More common are antigens that are presented by tumor cells and normal cells, and they are called tumor-associated antigens (TAAs). Cytotoxic T lymphocytes that recognize these antigens may be able to destroy the tumor cells before they prolifere or metastasize. Tumor antigens may also be on the surface of the tumor in the form of, for example, a mutated receptor, in which case they may be recognized by B cells.

VIII. Indications

0205] Disclosed herein are methods for treating a disease or condition in a subject in need thereof, comprising administering the bispecific antibodies disclosed herein.

0206] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody of Formula I, IA, II or IA. The condition or disease may comprise a cancer. The cancer may be selected from a prostate cancer, a myeloid leukemia, an epithelial cancer, an ovarian cancer, a cervical cancer, a breast cancer, a lung cancer, a kidney cancer, a colorectal cancer, a brain cancer, a thyroid cancer, an adrenal cancer, a pancreatic cancer, an endometrial cancer, a neuroendocrine cancer, a gastrointestinal cancer, a non-Hodgkin’s lymphoma, a skin cancer, a gastric cancer, a glioblastoma and an Ewing’s sarcoma. The lung cancer may be a small cell lung cancer. The ovarian cancer may be a stromal ovarian cancer. The thyroid cancer may be a medullary thyroid cancer. The skin cancer may be a melanoma. The skin cancer may be a neo-angiogenic skin cancer. The colorectal cancer may be an exocrine pancreatic cancer.

0207] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-HER2 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a breast cancer.

0208] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-EGFRvIII antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a glioma or a glioblastoma.

0209] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-CD1 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a multiple myeloma.

0210] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-CD1 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be an acute myeloid leukemia.

0211] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-CD3 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be an acute myeloid leukemia.

0212] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-HER2 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a B-cell chronic lymphocytic leukemia. The cancer may be a mantle cell lymphoma. The cancer may be an acute lymphoblastic leukemia.

0213] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-HER2 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a B-cell chronic lymphocytic leukemia. The cancer may be a mantle cell lymphoma. The cancer may be an acute myeloid leukemia.

0214] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-HER2 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a glioblastoma.

0215] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to a bevacizumab antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a multiple myeloma.

0216] Disclosed herein are methods of treating a disease or condition comprising administering a bispecific antibody comprising an anti-CD3 antibody or fragment thereof conjugated to an anti-HER2 antibody or fragment thereof to a subject in need thereof. The condition or disease may be a cancer. The cancer may be a multiple myeloma. The multiple myeloma may be a relapsed multiple myeloma. The multiple myeloma may be refractory. The leukemia may be acute myeloid leukemia. The leukemia may be B-cell chronic lymphocytic leukemia. The leukemia may be mantle cell leukemia. The leukemia may be acute lymphoblastic leukemia.

0217] In some instances, the one or more diseases comprise a cancer. The cancer may be a recurrent and/or refractory cancer. Examples of cancers include, but are not limited to, sarcomas, carcinomas, gliomas, lymphomas or leukemias. The cancer may be a breast cancer. The cancer may be a prostate cancer. The cancer may be a lung cancer. The cancer may be a glioma. The cancer may be a glioblastoma. The cancer may be a myeloma. The cancer may be a multiple myeloma. The multiple myeloma may be a relapsed multiple myeloma. The multiple myeloma may be refractory. The leukemia may be acute myeloid leukemia. The leukemia may be B-cell chronic lymphocytic leukemia. The leukemia may be mantle cell leukemia. The leukemia may be acute lymphoblastic leukemia.

0218] Sarcomas are cancers of the bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Sarcomas include, but are not limited to, bone cancer, fibrosarcoma, chondrosarcoma, Ewing’s sarcoma, malignant hemangiendothelioma, malignant schwannoma, bilateral vestibular schwannoma, osteosarcoma, soft tissue sarcomas (e.g., alveolar soft part sarcoma, angiosarcoma, cystosarcoma phylloides, dermatofibrosarcoma, desmoid tumor, epithelioid sarcoma, extraskeletal osteosarcoma, fibrosarcoma, hemangiopericytoma, hemangiopericytoma, Kaposi’s sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphosarcoma, malignant fibrous histiocytoma, neurofibrosarcoma, rhabdomyosarcoma, and synovial sarcoma).
Carcinomas are cancers that begin in the epithelial cells, which are cells that cover the surface of the body, produce hormones, and make up glands. By way of non-limiting example, carcinomas include breast cancer, pancreatic cancer, lung cancer, colon cancer, colorectal cancer, rectal cancer, kidney cancer, bladder cancer, stomach cancer, prostate cancer, liver cancer, ovarian cancer, brain cancer, vaginal cancer, vulvar cancer, uterine cancer, oral cancer, penile cancer, testicular cancer, esophageal cancer, skin cancer, cancer of the fallopian tubes, head and neck cancer, gastrointestinal stromal cancer, adenocarcinoma, cutaneous or intraocular melanoma, cancer of the anal region, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, cancer of the urethra, cancer of the renal pelvis, cancer of the ureter, cancer of the endometrium, cancer of the cervix, cancer of the pituitary gland, neoplasms of the central nervous system (CNS), primary CNS lymphoma, brain stem glioma, and spinal axis tumors. In some instances, the cancer is a skin cancer, such as a basal cell carcinoma, squamous, melanoma, nonmelanoma, or actinic (solar) keratosis.

In some instances, the cancer is a lung cancer. Lung cancer may start in the airways that branch off the trachea to supply the lungs (bronchi) or the small air sacs of the lung (the alveoli). Lung cancers include non-small cell lung carcinoma (NSCLC), small cell lung carcinoma, and mesothelioma. Examples of NSCLC include squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. The mesothelioma is a cancer of the lining of the lung and chest cavity (pleura) or lining of the abdomen (peritoneum). The mesothelioma may be due to asbestos exposure. The cancer may be a brain cancer, such as a glioblastoma.

In some instances, the cancer is a breast cancer. In some instances, the breast cancer is estrogen receptor positive, progesterone receptor positive and Her2 positive (triple positive). In some instances, the breast cancer is estrogen receptor negative, progesterone receptor negative and Her2 negative (triple negative). In some instances, the breast cancer is estrogen receptor positive and Her2 positive. In some instances, the breast cancer is estrogen receptor positive and Her2 negative. In some instances, the breast cancer is estrogen receptor negative and Her2 positive. In some instances, the breast cancer is metastatic.

Alternatively, the cancer may be a central nervous system (CNS) tumor. CNS tumors may be classified as gliomas or nongliomas. The glioma may be malignant glioma, high grade glioma, diffuse intrinsic pontine glioma. Examples of gliomas include astrocytomas, oligodendrogliomas (or mixtures of oligodendroglioma and astrocytoma elements), and ependymomas. Astrocytomas include, but are not limited to, low-grade astrocytomas, anaplastic astrocytomas, glioblastoma multiforme, pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma. Oligodendrogliomas include low-grade oligodendrogliomas (or oligoastrocytomas) and anaplastic oligodendrogliomas. Nongliomas include meningiomas, pituitary adenomas, primary CNS lymphomas, and medulloblastomas. In some instances, the cancer is a meningioma.

The leukemia may be an acute lymphocytic leukemia, acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, or chronic myelocytic leukemia. Additional types of leukemias include hairy cell leukemia, chronic myelomonocytic leukemia, B-cell chronic lymphocytic leukemia and juvenile myelomonocytic leukemia.

Lymphomas are cancers of the lymphocytes and may develop from either B or T lymphocytes. The two major types of lymphoma are Hodgkin’s lymphoma, previously known as Hodgkin’s disease, and non-Hodgkin’s lymphoma. Hodgkin’s lymphoma is marked by the presence of the Reed-Sternberg cell. Non-Hodgkin’s lymphomas are all lymphomas which are not Hodgkin’s lymphoma. Non-Hodgkin lymphomas may be indolent lymphomas and aggressive lymphomas. Non-Hodgkin’s lymphomas include, but are not limited to, diffuse large B cell lymphoma, follicular lymphoma, mucosa-associated lymphoid tissue lymphoma (MALT), small cell lymphocytic lymphoma, mantle cell lymphoma, Burkitt’s lymphoma, mediastinal large B cell lymphoma, Waldenström macroglobulinemia, nodal marginal zone B cell lymphoma (NMZL), splenic marginal zone lymphoma (SMZL), extranodal marginal zone B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, and lymphomatoid granulomatosis.

The one or more diseases or conditions may be a pathogenic infection. Pathogenic infections may be caused by one or more pathogens. In some instances, the pathogen is a bacterium, fungi, virus, or protozoan.

Exemplary pathogens include but are not limited to: Borettella, Barrella, Brucella, Campylobacter, Chlamydia, Chlamyphila, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Heliobacter, Legionella, Lepotospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema, Vibrio, or Yersinia. In some cases, the disease or condition caused by the pathogen is tuberculosis and the heterogeneous sample comprises foreign molecules derived from the bacterium Mycobacterium tuberculosis and molecules derived from the subject. In some instances, the disease or condition is caused by a bacterium is tuberculosis, pneumonia, which may be caused by bacteria such as Streptococcus and Pseudomonas, a foodborne illness, which may be caused by bacteria such as Shigella, Campylobacter and Salmonella, and an infection such as tetanus, typhoid fever, diphtheria, syphilis and leprosy. The disease or condition may be bacterial vaginosis, a disease of the vagina caused by an imbalance of naturally occurring bacterial flora. Alternatively, the disease or condition is a bacterial meningitis, a bacterial inflammation of the meninges (e.g., the protective membranes covering the brain and spinal cord). Other diseases or conditions caused by bacteria include, but are not limited to, bacterial pneumonia, a urinary tract infection, bacterial gastroenteritis, and bacterial skin infection. Examples of bacterial skin infections include, but are not limited to, impetigo which may be caused by Staphylococcus aureus or Streptococcus pyogenes; erysipelas which may be caused by a streptococcus bacterial infection of the deep epidermis with lymphatic spread; and cellulitis which may be caused by normal skin flora or by exogenous bacteria.

The pathogen may be a fungus, such as, Candida, Aspergillus, Cryptococcus, Histoplasma, Pneumocystis, and Stachybotrys. Examples of diseases or conditions caused by a fungus include, but are not limited to, jock itch, yeast infection, ringworm, and athlete’s foot.

The pathogen may be a virus. Examples of viruses include, but are not limited to, adenovirus, coxsackievirus,
Epstein-Barr virus, Hepatitis virus (e.g., Hepatitis A, B, and C), herpes simplex virus (type 1 and 2), cytomegalovirus, herpes virus, HIV, influenza virus, measles virus, mumps virus, papillomavirus, parainfluenza virus, poliovirus, respiratory syncytial virus, rubella virus, and varicella-zoster virus. Examples of diseases or conditions caused by viruses include, but are not limited to, cold, flu, hepatitis, AIDS, chickenpox, rubella, mumps, measles, warts, and poliomyelitis.

[0229] The pathogen may be a protozoan, such as Acanthamoeba (e.g., A. castellanii, A. culbertsoni, A. hatchetti, A. polyphaga, A. rhysodes, A. healyi, A. divionensis), Brachiola (e.g., B. conori, B. vesicularum), Cryptosporidium (e.g., C. parvum), Cyclospora (e.g., C. cayetanensis), Encephalitozoon (e.g., E. cuniculi, E. hellem, E. intestinalis), Entamoeba (e.g., E. histolytica), Esterocystozoon (e.g., E. beneuisci), Giardia (e.g., G. lamblia), Isospora (e.g., I. belli), Microsporidium (e.g., M. africanaum, M. ceylonensis), Naegleria (e.g., N. fowleri), Nosema (e.g., N. algaeae, N. octoarticulata), Pleistophora, Trachipleistophora (e.g., T. anthropophihara, T. hominis), and Vittaforma (e.g., V. cornae).

[0230] The disease or condition may be an autoimmune disease or autoimmune related disease. An autoimmune disorder may be a malfunction of the body’s immune system that causes the body to attack its own tissues. Examples of autoimmune diseases and autoimmune related diseases include, but are not limited to, Addison’s disease, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome (APS), autoimmune aplastic anemia, autoimmune hemolytic anemia, autoimmunity hepatitis, autoimmune myocarditis, Behçet’s disease, celiac sprue, Crohn’s disease, dermatomyositis, eosinophilic fasciitis, erythema nodosum, giant cell arteritis (temporal arteritis), Goodpasture’s syndrome, Graves’ disease, Hashimoto’s disease, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, juvenile arthritis, diabetes, juvenile diabetes, Kawasaki syndrome, Lambert-Eaton syndrome, lupus (SLE), mixed connective tissue disease (MCTD), multiple sclerosis, myasthenia gravis, pemphigus, polyarteritis nodosa, type I, II, & III autoimmune polyglandular syndromes, polymyalgia rheumatica, polymyositis, psoriasis, psoriatic arthritis, Reiter’s syndrome, relapsing polyarthritis, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren’s syndrome, sperm & testicular autoimmune, stiff person syndrome, Takayasu’s arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis, vasculitis, vitiligo, and Wegener’s granulomatosis.

[0231] The disease or condition may be an inflammatory disease. Examples of inflammatory diseases include, but are not limited to, aneuritis, amyloidosis, angiitis, ankylosing spondylitis, atherosclerosis, B-cell lymphoma, cancer, celiac disease, cholesterol gallbladder disease, chronic fatigue syndrome, Cogan’s syndrome, congenital heart disease, connective tissue disease, Crohn’s disease, cystic fibrosis, DeQuervain’s tendinitis, diabetes associated arthritis, diffuse idiopathic skeletal hyperostosis, disoid lupus, Ehlers-Danlos syndrome, familial Mediterranean fever, fasciitis, fibrositis/fibromyalgia, frozen shoulder, ganglion cysts, giant cell arteritis, gout, Graves’ Disease, HIV-associated rheumatic disease syndromes, hyperparathyroid associated arthritis, infectious arthritis, inflammatory bowel syndrome/irritable bowel syndrome, juvenile rheumatoid arthritis, lyme disease, Marfan’s Syndrome, Mikulicz’s Disease, mixed connective tissue disease, multiple sclerosis, myofascial pain syndrome, osteoarthritis, osteomalacia, osteoporosis and corticosteroid-induced osteoporosis, Paget’s Disease, palindromic rheumatism, Parkinson’s Disease, Plummer’s Disease, polymyalgia rheumatica, polymyositis, pseudogout, psoriatic arthritis, Raynaud’s Phenomenon/Syndrome, Reiter’s Syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, sciatica (lumbar radiculopathy), scleroderma, scurvy, sickle cell arthritis, Sjogren’s Syndrome, spinal stenosis, spondylolisthesis, Still’s Disease, systemic lupus erythematosus, Takayasu’s (Pulseless) Disease, Tendinitis, tennis elbow/golf elbow, thyroid associated arthritis, trigger finger, ulcerative colitis, Wegener’s Granulomatosis, and Whipple’s Disease.

IX. Immune Modulation

[0232] The antibodies disclosed herein may be used to modulate an immune response. Modulation of an immune response may comprise stimulating, activating, increasing, enhancing, or up-regulating an immune response. Modulation of an immune response may comprise suppressing, inhibiting, preventing, reducing, or down-regulating an immune response. For example, the antibodies may comprise an anti-CD3 antibody or antibody fragment that may bind to a first cell and a second antibody or antibody fragment that may bind to a second cell. Binding of the antibody to the first and second cell may result in modulation of an immune response. The first cell may be an immune cell. The immune cell may be a hematopoetic cell. The second cell may be an immune cell, healthy cell, cancer cell, bacteria, or virally-infected.

Examples

[0233] The following specific and non-limiting examples are to be construed as merely illustrative, and do not limit the present disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present disclosure to its fullest extent. All patents, patent applications, and publications cited herein are hereby incorporated by reference in their entirety.

Example 1

Expression of Anti-CD3 Fab Double Mutant in E. coli

[0234] UCHT1-Fab sequences were obtained from the literature and inserted into a pBAD vector behind the α2M signal sequence. The sites for unnatural amino acid incorporation (light chain threonine 109 [LC-Thr109], light chain serine 202 [LC-Ser202], heavy chain alanine 123 [HC-A123], and heavy chain lysine 138 [HC-Lys138]) were quickchanged (QuikChange® Stratagene) to TAG amber nonsense codon. For the double mutant, two residues (LC-Ser202 and HC-Lys138) were mutated to TAG amber nonsense codon. FIG. 1 depicts a ribbon diagram of the UCHT1-Fab fragment. The pBAD vector was co-transformed with pULTRA-pAEC (a vector containing orthogonal M. fannuense tRNA and ami- noacyl-tRNA synthetase specific for pAEC) in DH10B cells. The cells were grown in LB media (2 L) supplemented with 100 mg/ml ampicillin, 25 mg/ml chloramphenicol, and 1 mM pAEC at 37°C and 250 rpm. At OD_{600} 0.8, cells were induced with 0.2% arabinose and moved to 30°C for 20 hours at 270 rpm. Cells were harvested and proteins were extracted by
incubating with 150 mL of periplasmic lysis buffer (20% sucrose, 30 mM Tris, pH 8, 1 mM EDTA, and 0.2 mg/mL lysozyme) for 30 min at 37°C. Extracts were clarified by centrifugation (18000 rpm, 30 min), filtered through 0.22 micron filter, and loaded onto Protein G column (GE healthcare). The column was washed with 20 bed volumes of 50 mM NaOAc, pH 5.2, and proteins were eluted with 10 bed volumes of 0.1 M NaOAc, pH 2.8. The eluents were immediately neutralized by adding 10% of 1 M Tris, pH 8, and dialysed against PBS for long-term storage.

**Example 2**

Synthesis of Bispecific Antibodies Using Genetically Encoded Unnatural Amino Acids

[0235] Our strategy takes advantage of genetically encoded unnatural amino acids with orthogonal chemical reactivity relative to the canonical twenty amino acids to site-specifically modify antibody fragments. Specifically, we used an evolved tRNA/aminosyl-tRNA synthetase pair to site-specifically incorporate p-acetylphenylalanine (pAcF, FIG. 2A) at defined sites in each of two Fab fragments in response to an amber nonsense codon. The mutant Fab fragments were then selectively coupled by stable oxime formation using the alkoxy-amine termini of two bifunctional linkers (FIG. 2B). In a second step, the two Fab-linker conjugates were linked to each other in order to obtain the heterodimer through a copper-free [3+2] Huisgen-cycloaddition ("Click" reaction) (FIG. 2C). This approach has a number of advantages over recombinant technologies and conventional coupling chemistries. For example, the use of bioorthogonal chemistries produces homogenous, chemically-defined products; variable linker lengths and conjugation sites on the antibody may be easily optimized to ensure flexibility and good efficacy for each specific application; sequences from existing monoclonal antibodies may be directly adopted; and the modular approach allows easy and rapid synthesis for combinatorial generation of diverse heterodimers (antibodies, enzymes, cytokines, etc.)

[0236] The bifunctional linkers (50-fold molar equivalents) were then coupled to the pAcF-containing anti-HER2 Fab (5 mg/mL) in excellent yields (>95% by ESI-MS, FIG. 9A-J) in 100 mM acetic buffer pH 4.5 at 37°C for 16 hours. FIG. 3A-C depicts ESI-MS analysis of Fab fragments before linker and after linker conjugation. FIG. 3A depicts Herceptin Fab mutant (LS202X, X=pAcF); FIG. 3B depicts Herceptin Fab-(PEG)3-Az; FIG. 3C depicts Herceptin Fab-(PEG)4-Octyne; FIG. 3D depicts UCHT1 Fab mutant (HK138X, X=pAcF); and FIG. 3E depicts UCHT1 Fab-(PEG)5-Octyne. FIG. 3F-J depict deconvoluted mass spectrum of Fab fragments before and after linker conjugation. FIG. 3G depicts Herceptin Fab mutant (LS202X, X=pAcF); FIG. 3H depicts Herceptin Fab-(PEG)3-Az; FIG. 3I depicts Herceptin Fab-(PEG)4-Octyne; FIG. 3J depicts UCHT1 Fab mutant (HK138X, X=pAcF); and FIG. 3K depicts UCHT1 Fab-(PEG)4-Octyne.

[0237] Excess linker was removed by an Amicon 10K concentrator column (Millipore) or by size exclusion chromatography (Superdex-75, GE Healthcare). The two Fab-linker conjugates were separately buffer exchanged into PBS, pH 7.4, then mixed at a 1:1 ratio at 10 mg/mL and incubated at 37°C for the copper-free Click reaction. The reaction was monitored by SDS-PAGE, and a band at ~100 kDa was observed, corresponding to the molecular weight of the Fab dimer. After 48 hours, about 70% of starting material was consumed, and the homodimer was easily purified by size-exclusion chromatography (Superdex-200, GE Healthcare) from the unreacted Fabs.

**Example 3**

Synthesis of Anti-Her2/Anti-CD3 Heterodimer

[0238] pAcFphe was substituted at HC-K138 in the anti-anti-CD3 antibody, UCHT1. This site is distal to the antigen-binding site and, when conjugated to the LC-5202 mutant anti-HER2 Fab with the same polyethylene glycol linker used above, should be long and flexible enough to allow the resulting bispecific antibody to productively bind both a CD3 positive T-cell and the HER2 positive target cell simultaneously. UCHT1 Fab was expressed in E. coli by the same method as described for anti-HER2 Fab and in Example 2, and the cyclooctyne linker-modified anti-CD3 was prepared as described in Example 7 in >95% yield as confirmed by ESI-MS (FIG. 3E, 3J). The anti-CD3-Fab-cyclooctyne conjugate was then coupled to anti-HER2-Fab-azide conjugate as described in Example 7 in 70% percent yield (determined by SDS-PAGE and chromatographic separation) and purified from unreacted Fab monomers by Superdex-200 size exclusion column. FIG. 4A shows the SDS-PAGE of the anti-Her2/anti-CD3 heterodimer. FIG. 4B shows the size exclusion chromatography FPLC trace of the anti-HER2/antCD3 heterodimer.

**Example 4**

Binding Characterization of Anti-Her2/Anti-CD3 Heterodimer

[0239] FACS analysis of the Fab heterodimer showed affinity for both HER2-positive (SK-BR-3) and CD3-positive (Jurkat) cells with no cross-reactivity of anti-CD3 Fab and anti-Her2 Fab, respectively (FIG. 5A-B). FIG. 5A shows the FACS analysis of CD3-positive (Jurkat) cells treated with anti-k-PE (line 1), anti-CD3 Fab (line 2), anti-Her2 Fab (line 3) or anti-Her2/anti-CD3 heterodimer (line 4). FIG. 5B shows the FACS analysis of HER2-positive (SK-BR-3) cells treated with anti-k-PE (line 1), anti-CD3 Fab (line 2), anti-Her2 Fab (line 3) or anti-Her2/anti-CD3 heterodimer (line 4).

[0240] To demonstrate the simultaneous binding of the heterodimer to the two antigens, we visualized cross-linking of fluorescently labeled SK-BR-3 and Jurkat cells. Specifically, SK-BR-3 cells and Jurkat cells were first stained with Mito Tracker Red (Invitrogen) and carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), respectively. The labeled Jurkat cells were incubated with 100 nM of the anti-HER2/anti-CD3 heterodimer in RPMI media supplemented with 10% FBS (fetal bovine serum) at 4°C for 30 minutes, and excess conjugate was washed away before mixing the Jurkat cells with SK-BR3 cells. A 1:1 mixture of un conjugated anti-CD3 and anti-Her2 Fabs was used as a negative control under the same labeling conditions. The cells were incubated at 37°C for 6 hours, allowing the adherent SK-BR-3 cells to attach to the plate; excess Jurkat cells were removed by gentle washing with media. In the sample with the heterodimer, significantly more Jurkat cells (small light grey circles, highlighted by horizontal arrows) were bound to the SK-BR-3 cells (larger dark grey circles, highlighted by vertical arrows in FIG. 5C, no arrows in FIG. 5D) after washing compared to cells incubated with the mixture of unconjugated Fabs (FIG. 5C-D), confirming heterodimer-mediated cell-cell interaction. FIG. 5C
shows the overlay of SK-BR-3 cells and Jurkat cells treated with the anti-HER2/anti-CD3 Fab heterodimer. FIG. 5D shows the overlay of SK-BR-3 cells and Jurkat cells treated with a 1:1 mixture of unconjugated anti-CD3 and anti-HER2 Fabs.

Example 5

**In Vitro Cytotoxicity of Anti-Her2/Anti-CD3 Heterodimer**

[0241] Next, we demonstrated that the heterodimer could recruit T cells to kill the target cancer cells in an in vitro cytotoxicity assay. Human PBMCs (peripheral blood mononuclear cells) were purified with Ficoll (GE Healthcare) from fresh blood of healthy donors and mixed with target cells, HER2-transfected MDA-MB-435 (33), at an effector to target cell ratio of 10 to 1 (1x10⁵ to 1x10⁶ cells) in RPMI media supplemented with 10% FBS. Non-transfected MDA-MB-435 cells were used as an isogenic negative control. A 1:1 mixture of unconjugated anti-HER2 and anti-CD3 Fabs were used as an additional negative control to further demonstrate that cytotoxicity was due to bispecific antibody-based interactions. After incubation for 16 hours at 37° C., the amount of LDH (lactate dehydrogenase) was measured from lysed cells as an indicator of cytotoxicity (9). FIG. 6 shows the dose-dependent cytotoxicity with MDA-MB-435/HER2+ cells in the presence of human PBMCs and antibody heterodimer. Different concentrations of anti-HER2/anti-CD3 Fab heterodimer (circles, line 1), or a 1:1 mixture of unconjugated anti-Her2 Fab and anti-CD3 Fab (square, line 2) were incubated with MDA-MB-435/HER2+ cells. Heterodimer (upward triangle, line 3) or a mixture (downward triangle, line 4) of anti-HER2 Fab and anti-CD3 Fab were incubated with MDA-MB-435/HER2+ cells as negative controls. After 17 hours of incubation at 37° C. and 5% CO₂, cytotoxicity was measured by levels of LDH (lactate dehydrogenase) release from lysed cells using the Cytotox 96 Nonradioactive Cytotoxicity Assay Kit (Promega). In separate wells, MDA-MB-435/HER2+ or MDA-MB-435/HER2− cells with no PBMCs were incubated and lysed using the lysis buffer (provided in the assay kit) as maximum cytotoxicity controls. The absorbance at 490 nm was recorded using SpectraMax 250 plate reader (Molecular Devices Corp.). Percent cytotoxicity was calculated by:

\[
\text{% Cytotoxicity} = \left( \frac{\text{Absorbance}_{\text{no effector}} - \text{Absorbance}_{\text{no effector normal}}}{\text{Absorbance}_{\text{max effector}} - \text{Absorbance}_{\text{max effector normal}}} \right) \times 100
\]

[0242] As shown in FIG. 6, lysis of HER2 positive target cells was observed in a dose dependent manner only when the conjugated anti-HER2/anti-CD3 heterodimer was present (FIG. 6, circles, line 1); the half maximal effective concentration (EC₅₀) was ~20 PM. HER2-negative MDA-MB-435 cells were not affected by the heterodimer, and neither cell line was affected by the unconjugated Fab mixture. The percent cytotoxicity of the HER2 positive and HER2 negative cells is shown in Table 1.

<table>
<thead>
<tr>
<th>% cytotoxicity</th>
<th>HER2+</th>
<th>HER2+ unconjugated</th>
<th>HER2−</th>
<th>HER2− unconjugated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>0.00%</td>
<td>0.135</td>
<td>2.564</td>
<td>1.2786</td>
</tr>
<tr>
<td></td>
<td>0.0381</td>
<td>0.653</td>
<td>4.9723</td>
<td>-0.1421</td>
</tr>
<tr>
<td></td>
<td>0.1526</td>
<td>0.363</td>
<td>7.2453</td>
<td>1.5627</td>
</tr>
<tr>
<td></td>
<td>0.0104</td>
<td>-1.1444</td>
<td>2.6992</td>
<td>1.8469</td>
</tr>
<tr>
<td></td>
<td>2.4414</td>
<td>11.7705</td>
<td>4.2505</td>
<td>10.9391</td>
</tr>
<tr>
<td></td>
<td>39.0265</td>
<td>66.3359</td>
<td>5.2313</td>
<td>11.7915</td>
</tr>
<tr>
<td></td>
<td>156.2500</td>
<td>93.1829</td>
<td>4.9044</td>
<td>12.6388</td>
</tr>
<tr>
<td></td>
<td>625.0000</td>
<td>93.3464</td>
<td>4.5774</td>
<td>16.0534</td>
</tr>
</tbody>
</table>

[0243] Additionally, cell lysis and formation of aggregates could be directly observed under a microscope in the heterodimer-present HER2 positive cell sample, but not in any of the controls (FIG. 7). FIG. 7A shows the microscopic images from the cytotoxicity assay in HER2− cells treated with the 1:1 mixture of unconjugated anti-HER2 Fab and anti-CD3 Fab. FIG. 7B shows the microscopic images from the cytotoxicity assay in HER2+ cells treated with the anti-HER2/anti-CD3 Fab heterodimer. FIG. 7C shows the microscopic images from the cytotoxicity assay in HER2+ cells treated with the 1:1 mixture of unconjugated anti-HER2 Fab and anti-CD3 Fab. FIG. 7D shows the microscopic images from the cytotoxicity assay in HER2+ cells treated with the anti-HER2/anti-CD3 Fab heterodimer.

Example 6

**In Vivo Cytotoxicity of Anti-Her2/Anti-CD3 Heterodimer**

[0244] Since both anti-Her2 and anti-CD3 Fabs recognize human epithelia, human cancer cells and human T cells must be introduced into immuno-deficient NOD-SCID (NOD, CB17-Prkdc<sup>scid</sup>/Ncr.Crl) mice. Others have also reported better efficiency with NOD-SCID IL-2-gamma-knockout mice (34) or by supplementing with irradiation (35). The simplest model includes premixing and co-injecting the tumor cells and purified human PBMCs (36, 37). While, this works for a preventative treatment model, it is not ideal for treatment models. Tumor cells, like MDA-MB-435 Her2-transfected, often take 1-2 weeks to reach the 100 mm<sup>3</sup> tumor size that is often required for treatment models before administering drug. A complication is that hPBMCs have been reported to only remain in NOD-SCID mice for 2 weeks, potentially reducing the efficacy of the time drug administration begins (37). Others have explored intraperitoneal (IP) and intravenous (IV) injection of PBMCs to reconstitute a human immune system in the mouse (38, 39). This method is more difficult and often is only retroactively confirmed of correct reconstitution by performing histology on the spleen. For the anti-her2/anti-CD3 Fab heterodimer, we began with the simplest prevention model.

[0245] First, the best ratio of cancer and non-activated hPBMCs for premixing was determined. Nine female NOD-SCID mice were divided into three groups: 1:1 ratio (2x10<sup>6</sup> MDA-MB-435/Her2<sup>−</sup> cells, 2x10<sup>6</sup> hPBMCs); 1:2 ratio (2x10<sup>6</sup> MDA-MB-435/Her2<sup>−</sup> cells, 4x10<sup>6</sup> hPBMCs); and 1:5 (2x10<sup>6</sup> MDA-MB-435/Her2<sup>−</sup> cells, 1x10<sup>7</sup> hPBMCs). All
groups were inject with 1:1 ratio of premixed cells to Matrigel above the right shoulder by subcutaneous injection (SC). Tumor size were monitored by caliper measurements of length and width every 3-4 days and approximated by the formula:

\[ \text{Tumor volume} = \frac{\text{Length} \times \text{Width} \times \text{Depth}}{2} \]

[0246] The results showed that the tumors in the 1:1 ratio group grew extremely fast, tumors in the 1:2 ratio group began to grow around day 15, and the tumors in the 1:5 ratio group were suppressed (Fig. 8). Fig. 8 shows growth curves of MDA-MB-435/Her2' premixed with hPBMC and Matrigel. The ratios of cancer cells to hPBMCs were 1:1 (square), 1:2 (triangle), and 1:5 (x) with 3 NOD-SCID mice per group. Tumors in 1:1 group grew very fast, tumors in 1:2 group began to grow after 2 weeks, and tumors in 1:5 group did not grow at all. As predicted, having too many hPBMCs inhibits tumor growth. While 1:1 ratio of cancer cells to hPBMCs grew very rapidly, it would not be ideal as the tumor cells were likely to overtake the number of hPBMCs very rapidly before the T cells are able to sufficiently kill the tumor cells. Hence, a 1:2 ratio for this particular cancer cell line was used for premixed preventative studies.

[0247] Preventative in vivo xenograft model was conducted by co-injecting 2x10⁶ MDA-MB-435/Her2* tumor cells, 4x10⁶ nonactivated human PBMCs, and Matrigel by SC into female NOD-SCID mice. After 4 days, the mice were grouped for treatment with anti-Her2/anti-CD3 Fab heterodimer (n=7), unconjugated anti-Her2 Fab and anti-CD3 Fab (n=7), or PBS (n=6). Because the serum half-life of the heterodimer was found to be about 6-7 hours in rats (collaboration with Ambrx, Inc.), six daily doses were given at 1 mg/kg per dose IV. After 17 days, the treatment and anti-Her2/anti-CD3 Fab heterodimer had significant tumor reduction (P<0.05 by one-way ANOVA) compared to unconjugated Fabs and PBS control (Fig. 9). Fig. 9 shows the results for the preventative in vivo xenograft model with premixed MDA-MB-435/Her2' cancer cells (2x10⁶ cells), hPBMCs (4x10⁶ cells), and Matrigel. Treatment of anti-Her2/anti-CD3 Fab heterodimer (circle), unconjugated anti-Her2 and anti-CD3 (square=Fab mixture), or PBS (triangle) was given daily by IV starting on Day 4. Table 2 shows the average tumor volume for conjugate (anti-Her2/anti-CD3 Fab heterodimer), unconjugate (anti-Her2 and anti-CD3-Fab mixture), or PBS-treated mice.

<table>
<thead>
<tr>
<th>Day</th>
<th>Conjugate</th>
<th>Unconjugated</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[0248] While it is likely that there are very few tumor cells left in the anti-Her2/anti-CD3 Fab heterodimer group, the size did not reduce to zero. This is likely due to residual Matrigel, which often stays in the mice for 3-6 weeks after initial injection. Additionally, a small signal was still observed by IVIS imaging, but did show a drastic difference in size between heterodimer treatment and unconjugated groups (Fig. 10). Fig. 10 shows the IVIS imaging of two heterodimer treatment mice (left two) and two unconjugated Fabs mice (right two). Luciferin was injected into each mice and incubated for 10 minutes before capturing the image. The MDA-MB-435/Her2' were transfected to include the firefly luciferase gene. Luminescent units are relative. As shown in Fig. 10, ROI 1=6.084±08 (mouse #1), ROI 2=1.805±09 (mouse #2), ROI 3=7.751±09 (mouse #3), and ROI 4=6.380±09 (mouse #4).

[0249] The Matrigel may have formed a matrix where it protected a small amount of tumor cells from being killed or small population of Her2' or Her2 low cells in the cancer cell line had escaped death. The excised tumor at the end of Day 20 did confirm that the majority of the remaining lump in the treatment group was due to Matrigel, which is often soft and white while tumor is solid and red (due to blood vessels) (Fig. 11). Fig. 11 shows the excised tumor or Matrigel from the preventative anti-Her2/anti-CD3 Fab heterodimer study. The top row is the heterodimer treatment group (n=7), middle row is unconjugated Fabs group (n=7), and bottom row is the PBS group (n=6). The "tumors" left from the heterodimer treatment group is most likely residual Matrigel, which is characterized by being soft and white.

Example 7

Synthesis of Bipartition Linkers

[0250] All chemicals were obtained from commercial sources and used without further purification. 1H- and 13C-NMR spectra were obtained on a Varian NOVA-399 (400 MHz) or MER-300 (300 MHz) spectrophotometer. Chemical shift values were recorded as parts per million relative to tetramethylysilane as an internal standard. Protein mass spectra were acquired at the Scripps Center for Mass Spectrometry (La Jolla, Calif.).

[0251] Fig. 12 shows a general scheme for synthesizing bifunctional linkers. As shown in Fig. 12, Triphenylphosphine (430 mg, 1.64 mmol), N-hydroxysuccinimide (270 mg, 1.66 mmol) and DIAD (0.32 mL, 1.6 mmol) were added to a solution of 1-azido-3,6,9-trioxadecane-1-ol (300 mg, 1.37 mmol) in CH₂Cl₂ (20 mL) at 0°C. After stirring overnight at room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (Hex:EtOAc, 1:1) to afford compound 5 (426 mg, 85%). Rf 0.2 (Hex:EtOAc, 1:1). 1H NMR (400 MHz, CDCl₃); δ 7.82 (dd, J=5.4, 3.1 Hz, 2H), 7.74 (dd, J=5.5, 3.1 Hz, 2H), 4.38-4.34 (m, 2H), 3.88-3.82 (m, 2H),
3.69-3.62 (m, 4H), 3.62-3.54 (m, 6H), 3.36 (s, 1J = 5.6 Hz, 2H), 13C NMR (100 MHz, CDCl₃): δ 163.96, 134.98, 129.53, 124.02, 77.76, 71.34, 71.17, 71.17, 71.12, 70.55, 69.83, 51.23. MS (ESI) calc'd. for C₁₆H₁₂O₄NaO₆ (M⁺+Na) 387.1, found 387.1. The structure of compound 5 is shown as Formula V below.

As shown in FIG. 12, TEA (0.18 mL, 1.3 mmol) was added to a solution of (1R,8S,9R)-bicyclo[6.1.0]non-4-yn-9-ylmethy(4-nitrophenyl)carbonate (compound 6, 270 mg, 0.86 mmol) in DMF (10 mL). O,O'-oxybis(2,1-ethanediyloxy-2,1-ethanediyloxy)bis(hydroxyxymine) (7, 770 mg, 3.4 mmol) in CH₂Cl₂ (40 mL) was added to the solution and stirred overnight at room temperature. The solvent was removed by blowing nitrogen gas, and the residue was purified by flash column chromatography on silica gel (CH₂Cl₂-MeOH, 20:1) to afford 3 (280 mg, 82%). Rf 0.2 (CH₂Cl₂-MeOH, 10:1). 1H NMR (300 MHz, CDCl₃) δ 8.16 (s, 1H), 5.58 (br, s, 2H), 4.10-4.00 (m, 4H), 3.89-3.83 (m, 2H), 3.78-3.70 (m, 4H), 3.68 (s, 2H), 2.40 (d, J=13.6 Hz, 2H), 2.27 (d, J=13.7 Hz, 2H), 2.16 (d, J=16.1 Hz, 2H), 1.59-1.27 (m, 2H), 0.89-0.62 (m, 3H). 13C NMR (75 MHz, CDCl₃): δ 157.95, 98.98, 75.64, 74.99, 70.81, 70.73, 70.25, 69.84, 69.49, 33.46, 23.80, 23.24, 21.59. HRMS (ESI) calc'd. for C₁₃H₁₂N₃O₄ (M⁺+1) 401.2282, found 401.2287. The structure of compound 3 is shown as Formula VII below.

As shown in FIG. 12, 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)iodoindol-1,3-dione (compound 5, 426 mg, 1.17 mmol) was dissolved in methanolic ammonia solution (7N in MeOH, 50 mL) and stirred overnight at 40°C. After the solvent was removed under reduced pressure, the crude mixture was dissolved in CH₂Cl₂ (50 mL), and the white precipitate was filtered off. After concentration, the residue was purified by flash column chromatography on silica gel (CH₂Cl₂-MeOH, 50:1) to afford compound 2 (254 mg, 93%). Rf 0.3 (CH₂Cl₂-MeOH, 50:1). 1H NMR (400 MHz, CDCl₃) δ 3.90-3.82 (m, 2H), 3.70-3.67 (m, 4H), 3.66 (s, 6H), 3.67-3.65 (m, 2H), 3.39 (t, J=5.6 Hz, 2H). 13C NMR (100 MHz, CDCl₃): δ 74.70, 70.69, 70.62, 70.60, 70.52, 70.01, 69.66, 50.72. HRMS (ESI) calc'd. for C₁₃H₁₂N₃O₄ (M⁺+1) 235.1403, found 235.1403. The structure of compound 2 is shown as Formula VI below.

[0252] As shown in FIG. 12, 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)iodoindol-1,3-dione (compound 5, 426 mg, 1.17 mmol) was dissolved in methanolic ammonia solution (7N in MeOH, 50 mL) and stirred overnight at 40°C. After the solvent was removed under reduced pressure, the crude mixture was dissolved in CH₂Cl₂ (50 mL), and the white precipitate was filtered off. After concentration, the residue was purified by flash column chromatography on silica gel (CH₂Cl₂-MeOH, 50:1) to afford compound 2 (254 mg, 93%). Rf 0.3 (CH₂Cl₂-MeOH, 50:1). 1H NMR (400 MHz, CDCl₃) δ 3.90-3.82 (m, 2H), 3.70-3.67 (m, 4H), 3.66 (s, 6H), 3.67-3.65 (m, 2H), 3.39 (t, J=5.6 Hz, 2H). 13C NMR (100 MHz, CDCl₃): δ 74.70, 70.69, 70.62, 70.60, 70.52, 70.01, 69.66, 50.72. HRMS (ESI) calc'd. for C₁₃H₁₂N₃O₄ (M⁺+1) 235.1403, found 235.1403. The structure of compound 2 is shown as Formula VI below.

[0255] Hereceptin-Fab and UCHT1-Fab sequences were obtained from the literature and inserted into a pBAD vector behind the stl signal sequence. The sites for unnatural amino acid incorporation (LC-Ser202 for Her2 Fab, HC-Lys338 for UCHT1) were quickchanged (Stratagene) to TAG amber nonsense codon. The pBAD vector was co-transformed with PhEOL-pAcF (a vector containing orthogonal M. jannaschii tRNA and aminosyyl-tRNA synthetase specific for pAcF in DH10B cells. The cells were grown in LB media (2 L) supplemented with 100 mg/ml ampicillin, 25 mg/ml chloramphenicol, and 1 mM pAcF at 37°C and 250 rpm. At OD₅₉₀ 0.8, cells were induced with 0.2% arabinose and moved to 30°C for 16 hours at 270 rpm. Cells were harvested and proteins were extracted by incubating with 150 mL of periplasmic lysis buffer (20% sucrose, 30 mM Tris, pH 8, 1 mM EDTA, and 0.2 mg/mL lysozyme) for 30 min at 37°C. Extracts were clarified by centrifugation (18000 rpm, 30 min), filtered through 0.22 micron filter, and loaded onto Protein G column (GE healthcare). The column was washed with 20 bed volumes of 50 mM NaOAc, pH 5.2, and proteins were eluted with 10 bed volumes of 100 mM glycine, pH 2.8. The eluents were immediately neutralized by adding 10% of 1 M Tris, pH 8, and dialysed against PBS for long-term storage.
Example 9

Linker Conjugation and Protein Coupling Reaction

The antibodies (anti-HER2 Fab, anti-CD3 Fab) were buffer exchanged into 100 mM NaOAc, pH 4.5, and the concentrations were adjusted to ~5 mg/mL. The oxime ligation was conducted with 50 times molar excess of linkers, and the reaction was complete within 24 hours, as monitored by LC-MS. Excess linkers were removed by size filtration (Amicon 10K) or size exclusion column (Superdex 75) chromatography, the antibodies were buffer exchanged into PBS, pH 7.4, and the protein concentrations were adjusted to 10 mg/mL. Fab-Az conjugate and Fab-cyclooctyne conjugate were mixed at 1:1 molar ratio (~10 mg in total), and incubated for 2 days at 37°C. Dimeric Fab conjugate was purified from unreacted Fabs by size exclusion column (Superdex 200) (Fig. 4B).

Example 10

Flow Cytometry Analysis

SK-BR-3 cells were trypsinized (0.05% trypsin/EDTA, Hyclone) and washed with PBS. Jurkat cells in suspension were directly used without trypsinization. Cells (0.2 x 10⁶) were blocked with 3% BSA in PBS (200 ml) for 1 hour at 4°C, then incubated with 100 μM of primary antibodies for 1 hour at 4°C. Cells were washed (cold PBS 1 ml) twice, resuspended in 200 ml of cold PBS, and incubated with secondary goat anti-human kappa PE conjugated antibody (100X, Southern Biotech) for 30 min at 4°C. Cells were washed twice with 1 ml of cold PBS before analysis on a BD LSR II flow cytometer (Becton Dickinson Immunocytometry Systems) with 10,000 cell events per sample. Data was analyzed using FlowJo software (Tree Star Inc.).

Example 11

Fluorescent Staining

SK-BR-3 and Jurkat cells were stained with MitoTracker Red (Invitrogen) and CFSE (carboxyfluorescein succinimidyl ester, Invitrogen), respectively, following the manufacturer’s protocol. Jurkat cells (2 x 10⁶) were incubated with anti-HER2/anti-CD3 heterodimer (100 nM) in 200 μl of PBS for 30 min at 4°C. In a separate tube, Jurkat cells were incubated with a 1:1 mixture of anti-HER2 Fab and anti-CD3 Fab (100 nM each) as negative controls. After washing with 1 ml of cold PBS, the Jurkat cells were resuspended with 200 μl of RPMI media with 10% FBS, then mixed with SK-BR-3 cells (2 x 10⁶) in the same media (200 μL). Two hundred microliters of the cell mixture was added into a clear bottom 96 well plate, and incubated at 37°C and 5% CO₂. After 6 hours, wells were gently washed with PBS (200 μL) 4 times and imaged on a fluorescence microscope (Eclipse Ti, Nikon) under FITC (for CFSE) and rhodamine (for Mito Tracker Red) filters. The images from each filter were combined to produce an overlay image as seen in FIG. 7C.

Example 12

In Vitro Cytotoxicity Assays

Peripheral blood mononuclear cells (PBMCs) were purified from fresh healthy human donor blood by conventional Ficoll-Hypaque gradient centrifugation. Purified PBMCs were washed and incubated in flasks in RPMI media with 10% FBS for 2 hours to remove any adherent cells, and then transferred to anti-CD3 (eBioScience) and anti-CD28 (eBioScience) antibody-coated ELISA plates at 37°C. After 3 days, the PBMCs were transferred into a flask and incubated with 20 units/ml IL-2 (R&D Systems) for T cell proliferation. HER2-transfected MDA-MB-453 or non-transfected MDA-MB-435 cells (target cells) were dissociated with 0.05% trypsin/EDTA solution (HyClone) and washed with RPMI with 10% FBS, 1 x 10⁶ target cells were mixed with PBMCs at 1:1 ratio in 100 μl, and incubated with different concentrations of conjugated and unconjugated anti-Her2/anti-CD3 Fabs (10 μl in PBS) for 17 hours at 37°C. Cytotoxicity of each well was measured for LDH (lactate dehydrogenase) levels in supernatant using Cytoxtox-96 non-radioactive cytotoxicity assay kit (Promega). Lysis solution (10 μl, provided in the same kit) was added to wells with only target cells to get the maximum killing, and spontaneous killing was measured from wells with effector and target cells treated with vehicle (10 μl PBS). The absorbance at 490 nm was recorded using SpectraMax 250 plate reader (Molecular Devices Corp.). Percent cytotoxicity was calculated by:

\[
\% \text{ Cytotoxicity} = \frac{(\text{Absorbance}_{\text{test}} - \text{Absorbance}_{\text{cytotoxic control}})}{(\text{Absorbance}_{\text{unlabeled cytotoxic control}} - \text{Absorbance}_{\text{cytotoxic control}})} \times 100
\]

Example 13

In Vivo Xenograft Models

Preventative in vivo xenograft model of bispecific antibodies was conducted by implanting pre-mixed MDA-MB-435/Her2⁺ cells (2 x 10⁶), non-activated PBMC (4 x 10⁵), and Matrigel on the right shoulder of female NOD-SCID (NOD.CB17-Prkdc<sup>scid</sup>/NcrCrl) mice by SQ. After 2-4 days, mice were randomized and grouped into 6-7 mice per group and given compound by IV. For the BiFab conjugate, unconjugate, or PBS was given daily at 1 mg/kg for 6-10 days. Tumors were monitored twice a week by caliper measurements of length, width, and height and the tumor volume was estimated by: Tumor volume=(length x width x height)/2.

Example 14

Construction of IgG-Based Bispecific Antibodies

The first step of bispecific antibody construction using our method was conjugating the bifunctional linker to the respective antibody components. Linker bearing the azide was conjugated to anti-Her2 IgG and Fab, while the linker bearing cyclooctyne was conjugated to anti-CD3 Fab single (HC-K138X) and double mutant (LC-520XX, HC-K138X). The oxime ligation was conducted with 50-fold molar excess of linker in acetate buffer pH 4.5 at 37°C for 16-48 hours. Complete conjugation was monitored by LCMS of the anti-CD3 Fab conjugation reaction (IgG reaction would have
required additional preparation for LC/MS). Excess linker was removed by size exclusion chromatography or excess washing in 30 kDa or 10 kDa Amicon concentration columns (Millipore). Antibody-linkers were buffer exchanged into PBS for the second step of conjugation. For Durga, anti-Her2-IgG Az, and anti-CD3-Fab-Oct (single mutant) were incubated at 1:8 molar ratio together and concentrated to 10 mg/ml total. For Fidler, anti-Her2-IgG Az, was anti-CD3-Fab-Oct (double mutant) were incubated at 1:1 molar ratio at a final concentration of only 1 mg/ml to encourage intramolecular reaction over intermolecular reaction. The Podracer compound utilized anti-Her2-Fab-Az and anti-CD3-Fab-Oct (single mutant) at a 1:8 molar ratio at a final concentration of 10 mg/ml. All constructs were incubated at 37°C and monitored every other day by SDS-PAGE for reaction completion, which usually took about 7-10 days. All conjugates were purified by Superdex 200 size exclusion column at 0.2 ml/min with PBS+100 mM NaCl as the mobile phase. SDS-PAGE was used to analyze each 0.5 ml fraction and the most pure fractions were combined and used for subsequent testing. Purified conjugates were then further analyzed by non-reducing and reducing SDS-PAGE gels.

Example 15
Western Blot of Her2 Receptor Expression Levels

[0262] The Her2 expression level on different cell lines were determined by Western blot. 1x10⁶ Her2 positive cells (SK-BR-3, HCC-1954, MDA-MB-453/Her2+ and 1x10⁶ Her2 "negative" cells (MDA-MB-231, MDA-MB-453/Her2-, CHO) were washed once with PBS and resuspended in 30 μL of Cell Lytic M buffer (Sigma) and incubated at 4°C for 30 min. The solution was centrifuged and the supernatant was mixed with SDS loading buffer for SDS-PAGE. Semi-dry transfer to PVDF membrane (Invitrogen) was conducted, and the membrane was blocked with 3% milk (BioRad) in PBS with 0.01% Tween (blocking buffer) for 1 hr at room temperature. Primary anti-Her2-IgG antibody (the same one used for conjugation) was incubated at 1 μg/ml in blocking buffer at 4°C overnight. The membrane was washed 4 times with PBS+0.01% Tween for 10 min each. Anti-kappa-HRP secondary (Sigma) was then incubated at room temperature for an hour and washed 4 more times with PBS+0.01% Tween. SuperSignal West Durga Extended Duration Substrate (Thermo Scientific) was incubated with the membrane for 10 minutes, washed once with PBS+0.01% Tween, and developed on a SRX-101A developer (Konica Minolta) with Hybloc CL film (Denville Scientific).

Example 16
Expression of Proteins with Unnatural Amino Acids in E. coli

[0263] The general method for expressing proteins such as Z-domain, 14 lysozyme (14L) and LamB transmembrane protein were similar to that which was previously described (2). The gene of interest (with TAG in the codon position that is desired to be modified with the 12A) is usually on one plasmid and the desired tRNA and amino-acyl tRNA synthetase pair are on a different plasmid with a different selection marker and origin of replication. The most optimized system to date is the pEVL plasmid, which has one optimized tyrosyl-tRNA that responds to the amber codon and two copies of RS: one being a constitutive promoter to provide a basal level of expression and one behind the arabinose promoter for strong inducible expression. The pEVL plasmid (chloramphenicol resistance) is also very compatible with pET vector (ampicillin resistance), which is a common vector for protein expression but is restricted to BL21(DE3) cells. The co-transformed cells were grown in 2xYT or LB (required for LamB expression) at 37°C overnight or until saturation. The cultures were then inoculated at OD₆₀₀ of 0.2 into fresh media containing 1 mM UAA at 37°C. At OD₆₀₀ of 0.8-1.0, 1 mM IPTG and 0.02% arabinose were added and the cultures were moved to 30°C for overnight growth. The next day, cells were harvested for downstream application. Z-domain and T4L that contain C-terminal His6 tags were sonicated, clarified of precipitates, and incubated with Ni-NTA resin at room temperature for 30 min. Resin was washed twice in desktop centrifuge columns and eluted with buffer containing imidazole. For conjugation, protein was often buffer exchanged into PBS using Amicon centrifugal columns.

Example 17
Analysis of Anti-CD20/Anti-CD3 Bispecific Antibody

[0264] Anti-CD20 (for B cell lymphoma) and anti-CD3 Fab fragments and heterodimers were constructed according to methods as described in the previous examples. Briefly, we incorporated pAeF into anti-CD20 Fabs (S202pAeF), modified them with bifunctional linkers, and conjugated to anti-CD3 Fab to prepare anti-CD20 anti-CD3 bispecific antibody. All of the conjugation and purification steps followed the procedure described for the preparation of the anti-Her2/anti-CD3 bispecific antibody.

[0265] We also carried out in vitro cytotoxicity assays where we mixed target cells (e.g., Ramos cells) with hPBMCs at 1:10 ratio, and incubated with different concentrations of bispecific antibodies. Cytotoxicity was determined by detecting LDH from lysed cells as described in Example 17. FIG. 13 depicts a graph of the cytotoxicity of Ramos cells treated with an anti-CD20/anti-CD3 bispecific antibody (circle) or treated with unconjugated Fab fragments (square). Table 3 shows the cytotoxicity of Ramos cells treated with an anti-CD20/anti-CD3 bispecific antibody or treated with unconjugated Fab fragments.

**TABLE 3**

<table>
<thead>
<tr>
<th>Relative Fluorescence Units (RFU)</th>
<th>pM</th>
<th>AntiCD20/CD3 conjugated</th>
<th>unconjugated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.689</td>
<td>0.7075</td>
<td></td>
</tr>
<tr>
<td>0.038147</td>
<td>0.6775</td>
<td>0.6895</td>
<td></td>
</tr>
<tr>
<td>0.1525879</td>
<td>0.4935</td>
<td>0.7235</td>
<td></td>
</tr>
<tr>
<td>0.6103516</td>
<td>0.7175</td>
<td>0.723</td>
<td></td>
</tr>
<tr>
<td>2.441406</td>
<td>0.7075</td>
<td>0.7415</td>
<td></td>
</tr>
<tr>
<td>7.676255</td>
<td>0.7195</td>
<td>0.7205</td>
<td></td>
</tr>
<tr>
<td>39.0625</td>
<td>0.7535</td>
<td>0.685</td>
<td></td>
</tr>
<tr>
<td>156.25</td>
<td>0.8345</td>
<td>0.687</td>
<td></td>
</tr>
<tr>
<td>625</td>
<td>0.967</td>
<td>0.676</td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td>1.029</td>
<td>0.663</td>
<td></td>
</tr>
<tr>
<td>10000</td>
<td>1.13</td>
<td>0.6555</td>
<td></td>
</tr>
</tbody>
</table>
Example 18

Analysis of Anti-EGFR/Anti-CD3 Bispecific Antibody

[0266] Anti-EGFR (for KRAS/BRAF mutated colorectal or lung cancer) and anti-CD3 Fab fragments and heterodimers were constructed according to methods as described in the previous examples. Briefly, we incorporated pAeF into anti-EGFR Fabs (S202pAeF), modified them with bifunctional linkers, and conjugated to anti-CD3 Fab to prepare anti-EGFR/anti-CD3 bispecific antibody. All of the conjugation and purification steps followed the procedure described for the preparation of the anti-Her2/anti-CD3 bispecific antibody.

[0267] We also carried out in vitro cytotoxicity assays where we mixed target cells (AGS, A549, or HT29 cells) with hPBMCs at 1:10 ratio, and incubated with different concentrations of bispecific antibodies. Cytotoxicity was determined by detecting LDH from lysed cells as described in Example 17. FIG. 14 and Table 4 shows the cytotoxicity of A549 cells treated with an anti-EGFR/anti-CD3 bispecific antibody (circle) or treated with unconjugated Fab fragments (square). FIG. 15 and Table 5 shows the cytotoxicity of HT29 cells treated with an anti-EGFR/anti-CD3 bispecific antibody (circle) or treated with unconjugated Fab fragments (square). FIG. 16 and Table 6 shows the cytotoxicity of AGS cells treated with an anti-EGFR/anti-CD3 bispecific antibody (circle) or treated with unconjugated Fab fragments (square).

### Table 4

<table>
<thead>
<tr>
<th>pM</th>
<th>aEGFR/αCD3 conjugated</th>
<th>unconjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6004</td>
<td>1.3377</td>
<td>0.9040</td>
</tr>
<tr>
<td>2.4414</td>
<td>1.3780</td>
<td>0.8793</td>
</tr>
<tr>
<td>9.7556</td>
<td>1.3857</td>
<td>1.0233</td>
</tr>
<tr>
<td>39.9255</td>
<td>1.5187</td>
<td>0.9810</td>
</tr>
<tr>
<td>156.25</td>
<td>1.9837</td>
<td>0.9683</td>
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<tr>
<td>625</td>
<td>2.2673</td>
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<tr>
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<td>1.0847</td>
</tr>
<tr>
<td>10000</td>
<td>2.2860</td>
<td>1.2483</td>
</tr>
</tbody>
</table>

### Table 5

<table>
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<tr>
<th>pM</th>
<th>aEGFR/αCD3 conjugated</th>
<th>unconjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6004</td>
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<td>0.2333</td>
</tr>
<tr>
<td>2.4414</td>
<td>0.2447</td>
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<td>0.2500</td>
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<tr>
<td>625</td>
<td>0.3923</td>
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<tr>
<td>2500</td>
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<tr>
<td>10000</td>
<td>0.5380</td>
<td>0.2453</td>
</tr>
</tbody>
</table>

Example 19

Expression and Cytotoxicity of UCHT1-CS1 Bispecific Antibody

[0268] BiFab antibodies were created by expression of the UCHT1 Fab harboring a p-acetylphenylalanine (pAeF) residue in place of lysine at residue 138 (K138X). The protein was expressed in E. coli, purified by affinity column and conjugated to the Tet-TEG-ONH2 heterobifunctional linker via oxime ligation using previously reported conditions (buffer pH 4.5, 30-fold excess, overnight). The CS1 antibody was expressed in similar fashion using previously reported conditions. These antibodies harbored the pAeF residue in place of the serine 202 residue (S202X). The CS1 Fab was independently conjugated to TCO-TEG-ONH2 (subsequent generations were produced using a TCO linker (FIG. 2)). Conjugated CS1 was ligated to UCHT1-Tet Fab using previously reported "click" chemistry. Antibodies were purified over size-exclusion chromatography before testing in cytotoxicity assays.

Expression and Purification:

[0269] Fabs were expressed in E. coli as follows. Starter cultures of E. coli DH10B containing pBAD plasmid expressing the Fab and pUtra plasmid expressing the orthogonal tRNA/AARS pair for pAeF incorporation were grown overnight. Cultures were subsequently diluted to OD600~0.05, grown in the presence of 1 mM pAeF at 37° C with shaking at 250 rpm, and induced at OD600~1.0 with 0.2% arabinose and 1 mM IPTG. After induction, culture conditions were shifted to 26° C with shaking at 250 rpm, and the cells allowed to grow for 20-24 h. Cells were harvested at 5 k rpm for 30 min and cell pellets were frozen at ~80° C followed by thawing at room temperature. Cell pellets were resuspended in periplasmic lysis buffer (30 mM Tris-HCl, 1 mM EDTA, 20% sucrose pH 7.5 and 0.2 mg/mL lysozyme) and the cell suspension incubated at 37° C with shaking for 20-30 min. lysates were cleared at 14,000 rpm for 45 min and filtered through a 0.2 µm filter to remove particulates.

[0270] The cleared and filtered lysates were applied to pre-equilibrated columns packed with Protein G Sepharose resin (GE Healthcare), washed with 50 mM sodium acetate (pH 5.0), and protein eluted with 100 mM glycine (pH 2.8).

Conjugation:

[0271] Purified Fabs were buffer-exchanged into 100 mM sodium acetate (pH 4.5) 3 times using Amicon concentrators (10,000 MWCO). Fab (~5 mg/mL) was conjugated to the
appropriate heterobifunctional linker (TCO-TEG-ONH2 for UCHT1, and Tet-TEG-ONH2 for CS1) (FIG. 17B) at 50-fold excess linker to Fab, overnight at 37° C. (oxime ligation). The reactions werebuffer exchanged into PBS pH 7.5 and purified by size-exclusion chromatography (Superdex 200), and the products confirmed by mass spectrometry.

[0272] Coupling of CS1 Fab to UCHT1 Fab was carried out by mixing the two Fab-linkers in a 1:1 molar ratio and incubating at 37° C. for ~16 h. BisFab was purified using size-exclusion chromatography (Superdex 200), and the product confirmed by SDS-PAGE (FIG. 17A) and mass spectrometry analysis.

In Vitro Cytotoxicity:

LDH Assay

[0273] Peripheral blood mononuclear cells (PBMCs) were purified from fresh healthy donor blood (Scirps Clinical Research) by conventional Ficoll-Hypaque gradient centrifugation. Purified PBMCs were washed and incubated in flasks in RPMI 1640 medium with 5% FBS for 1 hour at 37° C., before washing with medium and adjusting concentration to 2x10^6 cells/ml. Target cells MML.S (CS1+) were washed with RPMI 1640 medium with 5% FBS and adjusted to a concentration of 0.2x10^6 cells/ml. Equal volumes of target cells and effectors were mixed together to obtain an effector:target ratio of 10:1, and dispensed in 100 uL volumes in a 96-well round-bottom plate. The effector-target mixtures were incubated with different concentrations of BisFab and unconjugated Fabs single or mixed (ranging from 10 nM down to 50 fM) for 24 hours at 37° C. Cytotoxicity of each well was measured as levels of LDH (lactate dehydrogenase) in supernatant using Cytox-tox-96 non-radioactive cytotoxicity assay kit (Promega). Lysis solution provided in the same kit, was added to wells with only target cells to get the maximum killing, and spontaneous killing was measured from wells with untreated effector and target cells. The absorbance at 490 nm was recorded using EnVision Multilabel Reader (Perkin Elmer). Percent cytotoxicity was calculated using the following formula: % Cytotoxicity = (Absorbance exp- Absorbance spontaneous average)/(Absorbance max killing average- Absorbance spontaneous average). FIG. 1C shows an increasing cytotoxicity as a result of increasing the concentration of the CS1-x/αCD3 BisFab.

Mass Spectrometry:

[0274] To analyze the mass of Fab’s and conjugates, a 10 uL of 0.1-1.0 mg/mL solution of Fab in PBS buffer was injected onto an LC Agilent 6520 Q-TOF mass spectrometer equipped with a 150x2.1 mm C8 column using a Water Acetonitrile (0.1% formic acid) gradient (20%-80% over 10 min). Briefly, electrospray settings were as follows: gas temp 350 deg C, drying gas 10 l/min, nebulizer 40 psi, fragmentor 200, VCap 4500, and TOF settings were standard (3200) range, 2 GHz, extended dynamic range. The region of the chromatogram in which the Fab eluted was deconvoluted using the Agilent Qualitative Analysis software with the pMod algorithm. FIG. 19 A-C shows the deconvolution results for unconjugated CS1 and UCHT1, CS1 and UCHT1 conjugated to their respective linkers, and the CS1-xUCHT1 bisFab, and Table 7 shows the summary of masses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected Mass</th>
<th>Obtained Mass</th>
<th>delta (obtained - expected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisFab</td>
<td>99696.77</td>
<td>99695.81</td>
<td>-0.96</td>
</tr>
<tr>
<td>CS1-xUCHT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS1-xEt</td>
<td>48329.68</td>
<td>48322.4</td>
<td>-7.28</td>
</tr>
<tr>
<td>UCHT1-TCO</td>
<td>48664.09</td>
<td>48665.62</td>
<td>1.33</td>
</tr>
<tr>
<td>CS1</td>
<td>47911.47</td>
<td>47902.74</td>
<td>-8.73</td>
</tr>
<tr>
<td>UCHT1</td>
<td>48355.88</td>
<td>48355.01</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Example 20

Expression and Cytotoxicity of Anti-CD33/Anti-CD3 and Anti-CLL-1/Anti-CD3 Bispecific Antibodies

Synthesis

[0275] Anti-CLL.1 and anti-CD3 Fab containing unnatural amino acid (pAcPhe) at heavy chain J38 position were expressed following the standard protocol. The Fab fragments were modified with hetero-bifunctional linkers and conjugated with anti-CD3 Fab, also following standard protocol described in the synthesis of other bispecific antibodies.

Flow Cytometry Analysis

[0276] HL60 (CD33 and CLL-1 positive) or CD3-positive Jurkat cells (0.2x10^6) were blocked with 3% BSA in PBS (200 ml) for 1 hour at 4° C, then incubated with 100 nM of primary antibodies for 1 hour at 4° C. Cells were washed (cold PBS 1 ml) twice, resuspended in 0.2 ml of cold PBS, and incubated with secondary goat anti-human kappa PE conjugated antibody (100X, Southern Biotech) for 30 min at 4° C. Cells were washed twice with 1 ml of cold PBS before analysis on a BD LSR II flow cytometer (Beckton Dickinson Immunocytometry Systems) with 10,000 cell events per sample. To get the FACS-based binding graph (FIG. 20A, anti-CLL.1/anti-CD3, FIG. 20B, anti-CD3/anti-CD3), the above procedure was repeated in round-bottomed 96-well format with different concentrations of primary antibodies. Cells were injected using high-throughput sampler (BD HTS). Data was analyzed using FlowJo software (Tree Star Inc.).

In Vitro Cytotoxicity Assay:

[0277] Purified PBMCs were washed and incubated in flasks in RPMI 1640 medium with 5% FBS for 1 hour at 37° C, before washing with medium and adjusting concentration to 2x10^6 cells/ml. HL60 cells were stained using PKH26 Red Fluorescent Cell linker kit (Sigma Aldrich) and the volume adjusted to give a final concentration of 0.2x10^6 cells/ml. Equal volumes of target cells and effectors were mixed together to obtain an effector:target ratio of 10:1, and dispensed in 100 uL volumes in a 96-well round-bottom plate. The effector-target mixtures were incubated with different concentrations of BisFab and unconjugated Fabs single or mixed for 24 hours at 37° C. After 24 hours, wells were treated with 7-aminoactinomycin D (7-AAD, BD Biosciences) for 30 min at room temperature to stain dead cells. For flow cytometry, unstained target cells, target cells stained with PKH26 only, and target cells stained with 7-AAD only...
(after treatment with ice-cold 90% methanol on ice for 30 min) were used as controls. Samples were read using LSRII Flow Cytometer (BD), and analyzed using FlowJo. Percentage viability was calculated as the ratio of live target cells in treated population to that in the untreated population (see FIG. 21A anti-CD33/anti-CD3 and FIG. 21B anti-CLL-1/anti-CD3).

**Cytokine Release Assay**

[0278] HL60 (CLL-1*CD33*) or RS4.11 (CLL-1*CD33*) cells were mixed with non-activated PBMCs (E:T=10:1), and treated with Bifabs at 25 nM for 24 hours. IFN-γ and IL-2 in supernatant were measured by ELISA (DuoSet ELISA Kit, R&D systems) (see FIG. 22A for IFN-γ and FIG. 22B for IL-2).

**TABLE 8**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Expected Mass</th>
<th>Observed Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCHT1/anti-CLL-1 bispecific antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCHT1/anti-CD3 pAcF</td>
<td>48400</td>
<td>48432</td>
</tr>
<tr>
<td>UCHT1/anti-CD33-Ar linker</td>
<td>48656</td>
<td>48653</td>
</tr>
<tr>
<td>UCHT1/anti-CLL-1 pAcF</td>
<td>48406</td>
<td>48407</td>
</tr>
<tr>
<td>UCHT1/anti-CLL-1-Ar linker</td>
<td>48712</td>
<td>48710</td>
</tr>
</tbody>
</table>

**Example 21**

**Expression and Cytotoxicity of Anti-EGFRvIII/anti-CD3 Bispecific Antibody**

[0279] BiFab antibodies were created by expression of the UCHT1 Fab harboring p-acetylated lysine (pAcF) residue in place of lysine at position 138 (K138X). The protein was expressed in *E. coli*, purified by affinity column and conjugated to the TCO-TEG-ONH2 heterobifunctional linker (FIG. 17B) via oxime ligation (buffer pH 4.5, 30-fold excess, overnight). Hu806 antibody was expressed in similar fashion using previously reported conditions. These antibodies harbored the pAcF residue in place of the serine 202 residue (S202X). Hu806 S202L C pAcF Fab was independently conjugated to Tet-TEG-ONH2 (FIG. 17B). Hu806-Tet Fab was then ligated to UCHT1-TCO Fab using "click" chemistry. Antibodies were purified over size-exclusion chromatography before testing in cytotoxicity assays.

**Expression and Purification:**

[0280] Fabs were expressed in *E. coli* as follows. Starter cultures of *E. coli* DH10B containing pBAD plasmid expressing the Fab and pUltrams plasmid expressing the orthogonal tRNA/AAARS pair for pAcF incorporation were grown overnight. Cultures were subsequently diluted to an OD600=0.05, grown in the presence of 1 mM pAcF at 37°C with shaking at 250 rpm, and induced at OD600=1.0 with 0.2% arabinose and 1 mM IPTG. After induction, culture conditions were shifted to 26°C with shaking at 250 rpm, and the cells allowed to grow for 20-24 hr. Cells were harvested at 5 k rpm for 30 min and cell pellets were frozen at −80°C, followed by thawing at room temperature. Cell pellets were resuspended in periplasmic lysis buffer (30 mM Tris-HCl, 1 mM EDTA, 20% sucrose pH 7.5 and 0.2 mg/ml lysozyme) and the cell suspension incubated at 37°C with shaking for 20-30 min. Lysates were cleared at 14,000 rpm for 45 min and filtered through a 0.2 μM filter to remove particulates. The cleared and filtered lysates were applied to pre-equilibrated columns packed with Protein G Sepharose resin (GE Healthcare), washed with 50 mM sodium acetate (pH 5.0), and protein eluted with 100 mM glyoxal (pH 2.8).

**Conjugation:**

[0281] Purified Fabs were buffer-exchanged into 100 mM sodium acetate (pH 4.5) 3 times using Amicon concentrators (10,000 MWCO). Fab (~5 mg/ml) was conjugated to the appropriate heterobifunctional linker (TCO-TEG-ONH2 for UCHT1, and Tet-TEG-ONH2 for hu806) at 50-fold excess linker to Fab, overnight at 37°C (oxime ligation). The reactions were buffer exchanged into PBS pH 7.5 and purified by size-exclusion chromatography (Superdex 200), and the products confirmed by mass spectrometry.

[0282] Coupling of hu806 Fab to UCHT1 Fab was carried out by mixing the two Fab-linkers in a 1:1 molar ratio and incubating at 37°C for 16 h. BiFab was purified using size-exclusion chromatography (Superdex 200), and the product confirmed by SDS-PAGE (FIG. 23) and mass spectrometry analysis (FIG. 24).

**In Vitro Cytotoxicity:**

[0283] Peripheral blood mononuclear cells (PBMCs) were purified from fresh healthy human donor blood (Scirpss Clinical Research) by conventional Ficoll-Hypaque gradient centrifugation. Purified PBMCs were washed and incubated in flasks in RPMI 1640 medium with 5% FBS for 2 hour at 37°C, before washing with medium and transferring to an anti-CD3 (eBioScience) coated flask (5 μg/ml). Anti-CD28 (eBioScience) was then added to the cell solution (2 μg/ml). After 3 days at 37°C, the PBMCs were transferred into a new flask and incubated with 20 units/ml IL2 (R&D Systems) for T cell proliferation.

[0284] Target cells A431 (EGFR*) were washed with RPMI 1640 medium with 5% FBS and adjusted to a concentration of 0.2×10⁶ cells/ml. Equal volumes of target cells and effectors were mixed together to obtain an effector:target ratio of 10:1, and dispensed in 100 μL volumes in a 96-well round-bottom plate. The effector:target mixtures were incubated with different concentrations of BiFabs and unconjugated Fabs single or mixed for 24 hours at 37°C. Cytotoxicity of each well was measured as levels of LDH (lactate dehydrogenase) in supernatant using Cytotox-96 non-radioactive cytotoxicity assay kit (Promega). Lysis solution provided in the same kit, was added to wells with only target cells to set the maximum killing, and spontaneous killing was measured from wells with untreated effector and target cells. The absorbance at 490 nm was recorded using EnVision Multilabel Reader (Perkin Elmer). Percent cytotoxicity was calculated using the following formula:

\[
\%\text{ Cytotoxicity} = \frac{\text{Absorbance exp} - \text{Absorbance spontaneous average}}{\text{Absorbance spontaneous average}} \times 100
\]
### TABLE 9

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected Mass</th>
<th>Obtained Mass</th>
<th>Delta (obtained – expected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifab</td>
<td>96275.34</td>
<td>96806.16</td>
<td>5311.82</td>
</tr>
<tr>
<td>Hu806-UCCH1</td>
<td>47625.25</td>
<td>47626.69</td>
<td>1.44</td>
</tr>
<tr>
<td>Tocixime</td>
<td>48664.08</td>
<td>48665.48</td>
<td>1.39</td>
</tr>
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<td>Hu806 Fab</td>
<td>47205.79</td>
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<td>0.3</td>
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<td>UCH1 Fab</td>
<td>48305.88</td>
<td>48306.65</td>
<td>0.75</td>
</tr>
</tbody>
</table>

### TABLE 10

**Sequences of antibodies or antibody fragments**

<table>
<thead>
<tr>
<th>SEQ</th>
<th>ID</th>
<th>NO</th>
<th>Description</th>
<th>SEQUENCE</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>of an anti-CD clone</td>
<td>of UCH1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>of an anti-</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>of UCH1</td>
</tr>
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<td></td>
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<td>of an anti-</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD clone</td>
<td>of UCH1</td>
</tr>
<tr>
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<td>of UCH1</td>
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<tr>
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<td></td>
<td></td>
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<td></td>
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<td>(anti-Her2)</td>
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**[0285]** The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

**[0286]** While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following define the scope of the invention and that methods and structures be within the scope of these claims and their equivalents be covered thereby.

What is claimed is:

1. A bispecific antibody comprising:
   a. an anti-CD3 antibody or anti-CD3 antibody fragment;
   b. a second antibody or antibody fragment, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically connected to the second antibody or antibody fragment.

2. The bispecific antibody of claim 1, wherein the anti-CD3 antibody or anti-CD3 antibody fragment is site-specifically connected to the second antibody or antibody fragment by one or more linkers.

3. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises an anti-EGFRvIII antibody.

4. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises an anti-Her2 antibody.

5. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises an anti-CS1 antibody.

6. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises an anti-CLL-1 antibody.

7. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises an anti-CD33 antibody.

8. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises an anti-CD20 antibody.

9. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment is selected from an anti-ROR1 antibody, an anti-CD44v6 antibody, an anti-PVRL4 antibody, an anti-IL-13Rα2 antibody and a bscWu1 antibody.

10. The bispecific antibody of claim 1, wherein the anti-CD33 antibody or anti-CD33 antibody fragment comprises one or more unnatural amino acids.

11. The bispecific antibody of claim 1, wherein the second antibody or antibody fragment comprises one or more unnatural amino acids.

12. The bispecific antibody of claim 1, wherein the anti-CD3 antibody or anti-CD3 antibody fragment and the second antibody or antibody fragment comprise one or more unnatural amino acids.
13. The bispecific antibody of claim 2, wherein the antibody is of Formula I: X-L1′-Y or Formula IA: Y-L1′-X, wherein:
   a. X comprises the anti-CD3 antibody or anti-CD3 antibody fragment;
   b. L1′ comprises the one or more linkers; and
   c. Y comprises the second antibody or antibody fragment.
14. The bispecific antibody of claim 13, wherein X is selected from a human, humanized, human engineered or fully human antibody.
15. The bispecific antibody of claim 1, wherein X comprises a chimeric antibody or portion thereof.
16. The bispecific antibody of claim 1, wherein X comprises a cross-species reactive antibody or a portion thereof.
17. The bispecific antibody of claim 16, wherein the anti-CD3 antibody is cross-species reactive with human and cynomolgus monkey.
18. The bispecific antibody of claim 13, wherein X and/or Y comprises one or more Fv, Fe, Fab, (Fab′)2, single chain Fv (scFv), diabodies, triabodies, tetabrodies, bifunctional hybrid antibodies, CDR1, CDR2, CDR3, combinations of CDR’s, variable regions, framework regions, constant regions, heavy chains, light chains, and variable regions, alternative scaffold non-antibody molecules, or a combination thereof.
19. The bispecific antibody of claim 13, wherein X and/or Y comprises a Fab fragment.
20. The bispecific antibody of claim 19, wherein the anti-CD3 antibody fragment is UCHT1.
21. The bispecific antibody of claim 13, wherein Y comprises at least a portion of a Fab fragment.
22. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody or antibody fragment that binds to an antigen on a hematopoietic cell.
23. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody or antibody fragment that binds to an antigen on a myeloid cell.
24. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody or antibody fragment that binds to an antigen on a lymphocyte.
25. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody or antibody fragment that binds to an antigen in a B-cell or B-cell progenitor.
26. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody or antibody fragment that binds to an antigen on a cancerous cell.
27. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody selected from the group comprising an anti-CD20 antibody, an anti-EGFR/HER2 antibody, an anti-CS1 antibody, an anti-CLL-1 antibody and an anti-CD33 antibody.
28. The bispecific antibody of claim 13, wherein Y comprises at least a portion of a Fab fragment of an antibody selected from the group comprising an anti-CD20 antibody, an anti-EGFR/HER2 antibody, an anti-CS1 antibody, an anti-CLL-1 antibody and an anti-CD33 antibody.
29. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an antibody selected from the group comprising an anti-ROR1 antibody, an anti-CD44v6 antibody, an anti-PVRL4 antibody, an anti-IL-13Rα2 antibody and an bscWue1 antibody.
30. The bispecific antibody of claim 13, wherein Y comprises at least a portion of Fab fragment of an antibody selected from the group comprising an anti-ROR1 antibody, an anti-CD44v6 antibody, an anti-PVRL4 antibody, an anti-IL-13Rα2 antibody and a bscWue1 antibody.
31. The bispecific antibody of claim 13, wherein Y comprises at least a portion of an anti-Her2 antibody.
32. The bispecific antibody of claim 13, wherein Y comprises at least a portion of a Fab fragment of an anti-Her2 antibody.
33. The bispecific antibody of claim 13, wherein Y is selected from SEQ ID NOs: 3-16.
34. The bispecific antibody of claim 13, wherein the bispecific antibody further comprises a second linker.
35. The bispecific antibody of claim 34, wherein the bispecific antibody is of Formula II: X-L1′-L2′-Y or Formula IIA: Y-L2′-L1′-X, wherein:
   a. a L1′ is coupled to X to produce a first intermediate of Formula III: X-L1′-X or Formula IIIA: X-L1′-X, wherein X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; and L1′ is coupled to a first linker before being coupled to X;
   b. L2′ is coupled to Y to produce a second intermediate of Formula IV: Y-L2′-Y or Formula IVA: L2′-Y; wherein Y comprises at least a portion of a second antibody or antibody fragment and L2′ comprises a second linker before being coupled to Y; and
   c. the first intermediate is coupled to the second intermediate.
36. The bispecific antibody of claim 35, wherein L1′ and/or L2′ comprises one or more chemical groups selected from an alkoxy-amine, hydrazine, ary1 azide, alkyl azide, alkyne, alken, tetrazine, dichlorotriazene, triazyl, succinimidyl carbonate, benzotriazole carbonate, nitrophenyl carbonate, trichlorophenyl carbonate, carbonylimidazole, succinimidyl succinate, maleimide, vinylsulfone, haloacetamide, cyclooctyne, trans-cyclooctene, cyclopentene, norborne and disulfide.
37. The bispecific antibody of claim 35, wherein one terminus of L1′ and/or one terminus of L2′ comprises an alkoxy-amine.
38. The bispecific antibody of claim 35, wherein one terminus of L1′ and/or one terminus of L2′ comprises an azide or cyclooctyne group.
39. The bispecific antibody of claim 35, wherein X is coupled to L1 by a covalent bond, ionic bond, or non-covalent bond.
40. The bispecific antibody of claim 35, wherein Y is coupled to L2 by a covalent bond, ionic bond, or non-covalent bond.
41. The bispecific antibody of claim 35, wherein X is coupled to L1 by an oxime.
42. The bispecific antibody of claim 35, wherein Y is coupled to L2 by an oxime.
43. The bispecific antibody of claim 35, wherein X-L1′ and L2′-Y are linked through a reaction selected from a Huisgen-cycloaddition, a Diels-Halden reaction, a hetero Diels-Alder reaction and an enzyme-mediated reaction.
44. The bispecific antibody of claim 43, wherein the enzyme-mediated reaction comprises a transglutaminase reaction.
45. The bispecific antibody of claim 35, wherein X-L1′ and Y-L2′ are linked through a copper-free [3+2] Huisgen-cycloaddition.
46. The bispecific antibody of claim 35, wherein X-L1′ and L2′-Y are linked through a disulfide or a maleimide.
47. The bispecific antibody of claim 35, wherein the distance between X and Y is less than about 50 angstrom (Å), about 45 angstrom (Å), about 40 angstrom (Å), about 35 angstrom (Å), about 30 angstrom (Å), about 25 angstrom (Å), about 20 angstrom (Å), about 15 angstrom (Å), about 10 angstrom (Å) or about 5 angstrom (Å).

48. The bispecific antibody of claim 2, wherein the one or more linkers comprises one or more ethylene glycol subunits.

49. The bispecific antibody of claim 13, wherein X and/or Y comprises one or more unnatural amino acids.

50. The bispecific antibody of claim 49, wherein one or more unnatural amino acids are site-specifically incorporated into X and/or Y.

51. The bispecific antibody of claim 49, wherein the one or more unnatural amino acids of X and/or the one or more unnatural amino acids of Y are genetically encoded.

52. The bispecific antibody of claim 51, wherein the one or more unnatural amino acids of X and/or the one or more unnatural amino acids of Y are genetically encoded by a codon selected from a nonsense codon, a stop codon, an amber codon, an ochre codon, an opal codon, a four-base codon and an amber codon.

53. The bispecific antibody of claim 49, wherein the one or more unnatural amino acids of X and/or the one or more unnatural amino acids of Y comprise a p-acetylphenylalanine (pAcF).

54. The bispecific antibody of claim 49, wherein the one or more unnatural amino acids of X and/or the one or more unnatural amino acids of Y comprise a selenocysteine.

55. The bispecific antibody of claim 49, wherein the site-specific connection occurs via the one or more unnatural amino acids of X and/or Y.

56. A method of producing a bispecific antibody, the method comprising connecting a plurality of anti-CD3 antibodies or anti-CD3 antibody fragments to a plurality of second antibodies or antibody fragments to produce a plurality of anti-CD3 bispecific antibodies, wherein at least 60% of the anti-CD3 bispecific antibodies are identical.

57. A method of producing a bispecific antibody of Formula II: X-L1'1°-L2°-Y or Formula IIIA: Y-L2°-L1'1°-X, comprising:
   d. coupling L1'1° to X to produce a first intermediate of Formula III: X-L11°-Y or Formula IIIA: L11°-X, wherein X comprises an anti-CD3 antibody or anti-CD3 antibody fragment; and L1'1° comprises a first linker before being coupled to X,
   e. coupling L2° to Y to produce a second intermediate of Formula IV: Y-L2° or Formula IIIA2: L2°-Y, wherein Y comprises at least a portion of a second antibody or antibody fragment and L2° comprises a second linker before being coupled to Y; and
   f. linking the first intermediate to the second intermediate, thereby producing the antibody of Formula II or IIIA.

58. The method of claim 57, wherein coupling L1'1° to X comprises site-specific coupling of L1'1° to X and/or coupling L2° to Y comprises site-specific coupling of L2° to Y.

59. The method of claim 57, further comprising incorporating one or more unnatural amino acids into X and/or Y.

60. The method of claim 59, wherein coupling L1'1° to X occurs at the one or more unnatural amino acids in X and/or coupling L2° to Y occurs at the one or more unnatural amino acids in Y.

61. The method of claim 59, further comprising modifying a nucleic acid encoding X and/or Y to produce one or more amber codons in X and/or Y.

62. The method of claim 57, wherein linking the first intermediate to the second intermediate comprises a Huisgen cycloaddition, a Diels-Halder reaction, a hetero Diels-Alder reaction or an enzyme-mediated reaction.

63. The method of claim 57, wherein linking the first intermediate to the second intermediate comprises a copper-free [3+2] Huisgen cycloaddition reaction.

64. The method of claim 57, wherein linking the first intermediate to the second intermediate produces an oxime, a tetrazole, a Diels Alder adduct, a hetero Diels Alder adduct, an aromatic substitution reaction product, a nucleophilic substitution reaction product, an ester, an amide, a carbamate, an ether, a thioether, a Michael reaction product, a cyclodaddition product, a metathesis reaction product, a metal-mediated cross-coupling reaction product, a radical polymerization product, an oxidative coupling product, an acyl-transfer reaction product, or a photo click reaction product.

65. The method of claim 57, wherein linking the first intermediate to the second intermediate produces a disulfide bridge or a maleimide bridge.

66. The method of claim 57, wherein the distance between X and Y is less than or equal to 50, 45, 40, 35, 30, 25, or 20 angstroms (Å).

67. The method of claim 57, wherein the distance between X and Y is greater than or equal to 5 angstroms (Å).

68. A pharmaceutical composition comprising the bispecific antibody of any of claims 1-54.

69. The pharmaceutical composition of claim 68, further comprising a pharmaceutically acceptable diluent, a pharmaceutically acceptable excipient or a pharmaceutically acceptable carrier.

70. A method for treating a disease or condition in a subject in need thereof, comprising administering the bispecific antibody of any of claims 1-55 or the pharmaceutical composition of any of claims 68 and 69.

71. The method of claim 70, wherein the disease or condition is a cancer.

72. The method of claim 71, wherein the cancer is a breast cancer.

73. The method of claim 71, wherein the cancer is a glioma or glioblastoma.

74. The method of claim 71, wherein the cancer is a multiple myeloma.

75. The method of claim 71, wherein the cancer is an acute myeloid leukemia (AML).

76. The method of claim 71, wherein the cancer is selected from an acute lymphoblastic leukemia (ALL), a B-cell chronic lymphocytic leukemia (B-CLL) and a mantle cell lymphoma (MCL).

77. The method of claim 70, wherein the disease or condition is a pathogenic infection.

78. The method of claim 70, wherein the disease or condition is an inflammatory disease, an autoimmune disease or a metabolic disease.

79. The method of claim 70, wherein the bispecific antibody or pharmaceutical composition is administered by parenteral administration.

80. The method of claim 79, wherein parenteral administration comprises intravenous administration, subcutaneous administration, intraperitoneal administration, intramuscular
administration, intravascular administration, intrathecal administration, intravitreal administration, or infusion.

81. The method of claim 70, wherein the bispecific antibody is administered by a microneedle device.

82. The method of claim 70, wherein the bispecific antibody is administered by topical, oral, or nasal administration.

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